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<p>(54) Title: REGENERATION AND TRANSFORMATION OF COTTON</p> <p>(57) Abstract</p> <p>There are provided methods for regenerating cotton by tissue and suspension culture starting with explants which are the hypocotyl, cotyledon or immature embryos. This also taught methods to transform cotton and improve cotton by selective growth.</p>			

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-1-

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REGENERATION AND TRANSFORMATION OF COTTON

BACKGROUND OF THE INVENTION

15 This invention is directed to plant regeneration and transformation of cotton, particularly cotton of the species Gossypium hirsutum L.

20 In recent years many tissues of diverse origin from plants belonging to different taxonomic groups have been established as in vitro tissue culture. Some of the factors controlling growth and differentiation of such cultures have also been determined. The establishment of subtle interactions among the different groups of plant hormones, and plant growth regulators operating either directly or indirectly, alone or in synergistic combination, have given to some degree an insight into certain 25 interrelationships that may exist among cells, tissues and organs. The information is however by no means complete.

30 For some time it has been known that plant cell cultures can be maintained in a nondifferentiating proliferative state indefinitely. It has, however, only been recently found that redifferentiation of tissues, organs or whole plant organisms can be experimentally induced. Since the demonstrations by Skoog et al "Chemical regulation of growth and organ formation in plant tissues 35 cultured in vitro," Symp. Soc. Exp. Biol. 11:18-130,

1 1958, incorporated herein by reference, that the relative
ratio of a cytokinin to an auxin determines the nature
of organogenesis in tobacco pith tissue. Reorganization
5 or regeneration from callus cultures includes the formation
of shoot primordia or embryos, both of which ultimately
lead to plantlet development in vitro.

10 The tendency for organogenesis vs. embryogenesis
still depends upon the species involved and the presence
of certain triggering factors which are chemical and/or
physical in nature.

15 In 1902, Haberlandt "Kulturversuche mit isolierten
pflanzenzellen," Mat. KI. Kais, Akad, Wiss. Wien 111:62,
incorporated herein by reference, postulated that plant
cells possessed the ability to produce entire plants and
predicted that this would someday be demonstrable in
cell cultures. In 1965, Reinert "Untersuchungen über
20 die Morphogenese an Gewebekulturen," Ber. dt. Bot. Ges.
71:15, and Steward et al, "Growth and organized development
of cultured cells/II. Organization in cultures grown
from freely suspended cells," Am. J. Bot. 45:705-708,
working independently, confirmed the occurrence of in
vitro somatic embryogenesis. Both references are
incorporated herein by reference. In experimentally
manipulating somatic embryogenesis it is believed that
25 two components of the culture media, an auxin and the
nitrogen source, play crucial roles.

30 It has also been shown that the process of somatic
embryogenesis takes place in two stages: first, the
induction of cells with embryogenic competence in the
presence of a high concentration of auxin; and second,
the development of embryonic cell masses into embryos in
the absence of or at a low concentration of auxin.

35 The induction of organogenesis or embryogenesis
leads to distinct structural patterns in the callus.
Detailed study of several plant species has enabled

1 certain generalizations to be made about the developmental
pathways leading to shoot, bud or embryo development.

5 The application of tissue culture techniques to the
regeneration of plants via organogenesis or embryogenesis
remains perhaps the most important contribution of basic
studies in morphogenesis to commercial application.

10 Beasley reported the formation of callus in ovule
cultures of cotton in 1971, "In vitro culture of ferti-
lized cotton ovules," Bioscience 21:906:907, 1971, incor-
porated herein by reference. Later, Hsu et al "Callus
induction by (2-chlorethyl) phosphonic (CPA) acid in
cultured cotton ovules," Physiol. Plant 36:150-153,
1976, incorporated herein by reference, observed a
stimulation of growth of calli obtained from ovules due
15 to the addition of CPA and gibberellic acid to the medium.
Callus cultures from other explants such as (a) leaf
Davis et al., "In vitro culture of callus tissues and
cell suspensions from okra (Hibiscus esculentus) and
cotton (Gossypium hirsutum)," In vitro 9:395-398, 1974,
20 both incorporated herein by reference; (b) hypocotyl
Schenk et al. "Medium and technique for induction and
growth of monocotyledonous and dicotyledonous plant cell
cultures," Can. J. Bot. 50:199-204, 1972, incorporated
herein by reference; and (c) cotyledons Rani et al.
25 "Establishment of Tissue Cultures of Cotton," Plant Sci. Lett. 7:163-169, 1976, incorporated herein by reference)
have been established for Gossypium hirsutum and G. arboreum.

30 Katterman et al, "The influence of a strong reducing
agent upon initiation of callus from the germinating
seedlings of Gossypium barbadense," Physiol. Plant 40:98-
101, 1977, incorporated herein by reference, observed
that the compact callus from cotyledons of G. barbadense
35 formed roots, and in one instance regeneration of a
complete plant was also obtained. Smith et al "Defined

1 conditions for the initiation and growth of cotton callus
in vitro, Gossypium arboreum," In vitro 13:329-334,
1977, incorporated herein by reference, determined
conditions for initiation and subculture of hypocotyl-
derived callus of G. arboreum. Subsequently, Price et
al "Callus cultures of six species of cotton (Gossypium
L) on defined media," Pl Sci. Lett. 8:115-119, 1977, and
"Tissue culture of Gossypium species and its potential
in cotton genetics and crop improvement," Beltwide Cotton
10 Production Research Conference Proc. pp. 51-55, 1977, of
the National Cotton Council, Memphis, each incorporated
herein by reference, defined conditions for the initiation
and subculture of callus from five species of Gossypium.

One of the common problems in establishing cultures
15 of many plant species is the "browning" of the explant
in the culture medium. In cotton, this leaching of
polyphenols was overcome by replacing sucrose with glucose,
and by transferring the cultures to a fresh medium every
10 days. After 3 or 4 passages on glucose supplemented
20 medium, the browning completely disappeared and the
cultures could be transferred back to sucrose-supple-
mented media. Although difficulties with the induction,
browning and maintenance of calli during subcultures
have been overcome with certain Gossypium species, all
25 attempts to regenerate plants from callus cultures have
been either unsuccessful or have involved several time-
consuming steps. Davidonis and Hamilton "Plant
Regeneration from Callus Tissue of Gossypium hirsutum,"
L. Plant Sci. Lett. 32:89-93, 1983, incorporated herein
30 by reference, reported the eventual formation of embryos
two years after the initiation of culture.

Although many growth substances, such as natural
phytohormones and synthetic growth regulating compounds
have been utilized in tissue culture media to bring
35 about plant regeneration in vitro, no generalization,

-5-

1 much less specifics, of the effects of different substances
on plant regeneration has been arrived at. Indeed, the
same substances, when applied to different plant species,
may either inhibit growth, enhance growth, or have no
5 effect whatsoever. Therefore, aside from certain standard
procedures, it remains necessarily a difficult task to
arrive at a working protocol for plant regeneration for
any new species and by many orders of magnitude a more
difficult task to achieve plant transformation.

10 The present invention provides a method for the
rapid regeneration of cotton plants from segments excised
from seedlings. The method described offers a high degree
of repeatability and reliability and it enables genetic
transformation of cotton plants.

15

Summary of the Invention

There is provided methods for the regeneration,
with optional transformation, of a cotton plant from
somatic cells.

20 Seed is sterilized and grown in the dark to a
seedling. The seedling is one source of an explant,
usually the hypocotyl and the cotyledon. Another source
are immature zygotic embryos of developing fruit. The
explant is subdivided and cultured in a first callus growth
25 medium (containing glucose) for a period of time to
allow a callus to develop from the explant on a culture
medium which copes with phenolic secretions and stimulates
cells of the explant to divide and proliferate. The
callus, after passing through the phenolic secretion
30 stage, is transferred to a fresh callus growth medium
(containing sucrose) which develops the callus to an
embryogenic callus. The embryo may then be subcultured
to produce more embryogenic callus or transferred to
another growth medium (plant germination medium) and
35 cultured for a period of time sufficient to develop a

1 plantlet which after another period of growth is transferred to a greenhouse, then into the field and grown to a mature plant from which seeds can be harvested.

5 The embryos may also be cultured in suspension. In this procedure, after the period of growth, the embryo containing embryogenic clumps greater than about 600 microns, preferably greater than about 800 microns in size are isolated and utilized for plant production. Smaller callus are recycled to the callus growth medium
10 for growth to plant forming callus or maintained as an embryos source.

15 Transformation may occur at the explant, callus or suspension development stage. Transformation involves exposing the explant, callus and/or embryogenic callus to the action of an Agrobacterium vector containing an expressible gene sequence foreign to cotton for a time sufficient for the gene to transfer into the cells. The residual Agrobacterium is then killed off with an antibiotic which is toxic to the Agrobacterium. This is
20 followed by selection of the transformed cells and/or embryogenic callus for development into transformed plantlets. In suspension culture, transformation and/or selection can occur prior to or following separation of embryogenic callus from cells and callus too immature to
25 be embryogenic.

30 Plants of unique phenotypic traits are obtainable, and there is provided new cotton plants which having resistance to antibiotics normally inhibitory to plant cell growth; cotton plants which have increased resistance or tolerance to herbicides, fungal pathogens and cotton plants which exhibit better yield and improved fiber quality.

1 Brief Description of the Drawings

5 FIG. 1 presents diagrammatically preferred procedures for development of cotton plants from seed by tissue culture techniques with a showing of establishing zones of transformation.

10 FIG. 2 is a photo illustration of embryogenic callus (10) of cotton with somatic embryos (12) at various stages of development including leaf (14) and root (16).

15 FIG. 3 is a photo illustration of a somatic cotton embryo at a late globular stage isolated to form the embryogenic callus culture as depicted in FIG. 2.

20 FIG. 4, as with reference to FIG. 2, is a photo illustration of embryos and young plantlets (18) of cotton developing on an embryo germination medium.

25 FIG. 5 is a photo illustration of small clumps of embryogenic cells from suspension cultures of cotton.

30 FIG. 6 is a photo illustration of a globular stage embryo from a suspension culture.

35 FIG. 7 illustrates germinating embryos obtained from suspension cultures showing emerging leaves (14) and roots (16).

40 FIG. 8 illustrates the development of plantlets of cotton growing on the embryo germination medium.

45 FIGS. 9 to 15 depict the genetic transformation of cotton, with FIG. 9 showing the development of cell colonies (20) from transformed cotton cells containing a gene for kanamycin resistance.

50 FIG. 10 shows somatic embryos developing from the selected antibiotic resistance cells of FIG. 9 on an antibiotic-supplemented medium.

55 FIG. 11 shows germinating embryos of transformed somatic embryos containing a gene conferring resistance to the herbicide glyphosate.

60 FIG. 12 shows cotton plantlets developed from the embryos of FIG. 11.

1 FIG. 13 shows germinating somatic embryos transformed
to confer resistance to Lepidopterous insects with leaf
14 and root 16 development.

5 FIG. 14 shows plantlets developed from the embryos
of FIG. 13.

FIG. 15 shows a plantlet of the variety Siokra
developed from transformed embryos exhibiting a resistance
to kanamycin.

10 FIG. 16 shows the construction of mp 19/bt, a plasmid
containing the 5' end of the Bt protoxin gene.

FIG. 17 shows the construction of mp 19/bt ca/del,
a plasmid containing the CaMV gene VI promotor fused to
the 5' end of Bt protoxin coding sequence.

15 FIG. 18 shows the construction of p702/bt, a plasmid
having the 3' coding region of the protoxin fused to the
CaMV transcription termination signals.

FIG. 19 shows the construction of pBR322/bt 14,
containing the complete protoxin coding sequence flanked
by CaMV promotor and terminator sequences.

20 FIG. 20 shows the construction of pRK252/Tn903/BglIII.

FIG. 21 shows the construction of PCIB 5.

FIGS. 22 & 23 shows the construction of pcIB 4.

FIG. 24 shows the construction of pcIB 2.

25 FIG. 25 shows the construction of pcIB 10, a broad
host range plasmid containing T-DNA borders and gene for
plant selection.

FIG. 26 shows the construction of pCIB10/19Sbt.

FIG. 27 shows the construction of pCIB 710.

FIG. 28 shows the construction of pCIB10/710.

30 FIG. 29 shows the construction of pCIB10/35Sbt.

FIG. 30 shows the construction of pCIB10//35Sbt(KpnI).

FIG. 31 shows the construction of pCIB10/35Sbt(BclI).

FIG. 32 shows the construction of pCIB10/35Sbt(607)

1 FIG. 33 depicts the vector DEI PEP10.

2 FIG. 34 is a photo showing a field trial made up of
3 cotton regenerants planted in a Verticillium infested
4 field.

5 FIG. 35 is a photo showing progeny of a regenerated
6 SJ4 plant in the field trial shown in FIG. 33. A
7 somaclonal variant with improved tolerance to Verticillium
8 fungus is indicated by the arrow.

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1 Detailed Description

5 The present invention is directed to the regeneration by tissue culture of cotton plants particularly plants of the genus Gossypium hirsutum from somatic cells for propagation in the field. Optionally, the cells may be transformed to include foreign genetic information.

The various growth medium useful in accordance with this invention are as follows:

10

SEED GERMINATION GROWTH MEDIUM

COMPOSITION OF MODIFIED WHITE'S STOCK SOLUTION
(*Phytomorphology* 11:109-127, 1961
incorporated herein by reference)

15	<u>Component</u>	<u>Concentra- tion per 1000 ml.</u>	<u>Comments</u>
20	MgSO ₄ .7 H ₂ O	3.6 g	Dissolve and make up the final volume to 1000 ml. Label <u>White's A Stock</u> . Use 100 ml/l of final medium.
	Na ₂ SO ₄	2.0 g	
	NaH ₂ PO ₄ .H ₂ O	1.65 g	
25	Ca(NO ₃) ₂ .4 H ₂ O	2.6 g	Dissolve and make up the final volume to 1000 ml. Label <u>White's B Stock</u> . Use 100 ml/l of final medium.
	KNO ₃	800 mg	
	KCl	650 mg	
30	Na ₂ MoO ₄ .2H ₂ O	2.5 mg	Dissolve and make up the final volume to 100 ml. Label <u>White's C Stock</u> . Use 1.0 ml/l of final medium.
	CoCl ₂ .6H ₂ O	2.5 mg	
	MnSO ₄ .H ₂ O	300 mg	
	ZnSO ₄ .7 H ₂ O	50 mg	
	CuSO ₄ .5 H ₂ O	2.5 mg	
	H ₃ BO ₃	50 mg	
	Fe EDTA		Use 10 ml/l of MSFe EDTA.
35	Organic		Use 10 ml/l of MS organic.

1

CALLUS GROWTH/MAINTENANCE MEDIUM

5

COMPOSITION OF MURASHIGE & SKOOG (MS)
STOCK SOLUTIONS
(Physiol. Plant 15:473-497, 1962
incorporated herein by reference)

	<u>Component</u>	<u>Concentration per 1000 ml. of Stock</u>	<u>Comments</u>
10	NH ₄ NO ₃	41.26 g	Dissolve and make up the
	KNO ₃	47.50 g	final volume to 1000
	CaCl ₂ · 2 H ₂ O	11.00 g	ml. Use 40 ml/l of final
	MgSO ₄ · 7 H ₂ O	9.25 g	medium.
	KH ₂ PO ₄	4.25 g	
15	KI	83 mg	Dissolve and make up
	H ₃ BO ₃	620 mg	the final volume to
	MnSO ₄ · H ₂ O	1690 mg	1000 ml. Label <u>MS -</u>
	ZnSO ₄ · 7 H ₂ O	860 mg	<u>Minor</u> . Use 10 ml/l of
	Na ₂ MnO ₄ · 2 H ₂ O	25 mg	final medium.
	CuSO ₄ · 5 H ₂ O	2.5 mg	
	CoCl ₂ · 6 H ₂ O	2.5 mg	
20	Nicotinic acid	50 mg	Dissolve and make up
	Pyridoxin HCl	50 mg	the final volume to
	Thiamine HCl	10 mg	1000 ml. Label <u>MS -</u>
			<u>Organic</u> . Freeze in 10
25			ml aliquots. Use 10
			ml/l of final medium.
30			
35			

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Fe EDTA
Fe SO₄.7H₂O
Na₂ EDTA.2 H₂O

2.78 g
3.73 g

Dissolve 2.78 g of FeSO₄.7 H₂O in about 200 ml of deionized water. Dissolve 3.73 g of Na₂ EDTA.2 H₂O (disodium salt of ethylenediaminetetra-acetic acid dihydrate) in 200 ml of deionized water in another beaker. Heat the Na₂ EDTA solution on a hot plate for about 10 minutes. While constantly stirring, add FeSO₄ solution to Na₂ EDTA solution. Cool the solution to room temperature and make up the volume to 1000 ml. Label MS EDTA. Cover bottle with foil and store in refrigerator. Use 10 ml/l of final medium.

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Thiamine HCl

50 mg

Dissolve and make up the volume to 500 ml. Label MS - Thiamine. Use 4.0 ml/l of final medium.

As if required.

25

Inositol
Glycine

10 g
0.2 g

Dissolve and make up the final volume to 1000 ml. Label MS - glycine/inositol. Use 10 ml/l of final medium.

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PLANT GERMINATION MEDIUM

COMPOSITION OF BEASLEY AND TING'S STOCK SOLUTIONS.

(Am. J. Bot. 60:130-139, 1973
incorporated herein by reference)

5

Concen-
tration
per 1000
ml.

Comments

10	<chem>KH2PO4</chem>	2.72	g	Dissolve and make up the volume to 100 ml.
	<chem>H3BO3</chem>	61.83	mg	Label <u>B&T - A Stock</u> .
	<chem>Na2MoO4.2 H2O</chem>	2.42	mg	Use 10 ml/l of final medium.
15	<chem>CaCl2.2 H2O</chem>	4.41	g	Dissolve and make up the volume to 100 ml.
	<chem>KI</chem>	8.3	mg	Label <u>B&T - B Stock</u> .
	<chem>CoCl2.6 H2O</chem>	0.24	mg	Use 10 ml/l of final medium.
20	<chem>MgSO4.7 H2O</chem>	4.93	g	Dissolve and make up the volume to 100 ml.
	<chem>MnSO4.H2O</chem>	169.02	mg	Label <u>B&T - C Stock</u> .
	<chem>ZnSO4.7 H2O</chem>	86.27	mg	Use 10 ml/l of final medium.
	<chem>CuSO4.5 H2O</chem>	0.25	mg	
25	<chem>KNO3</chem>	25.275	g	Dissolve and make up the volume to 200 ml.
	Nicotinic acid	4.92	mg	Label <u>B&T - D Stock</u> .
	Pyridoxin HCL	8.22	mg	Use 40 ml/l of final medium.
	Thiamine HCL	13.49	mg	
30	Fe EDTA			Dissolve and make up the final volume to 100 ml. Label <u>B&T -</u> <u>Organics</u> . Use 10 ml/l of final medium.
	Inositol			Use 10 ml/l of MS Fe EDTA.
	<chem>NH4NO3</chem> (15 uM)			100 mg/l of final medium.
35				1200.6 mg/l of final medium.

1 With any of the above solutions, the following
procedure is used to prepare one liter of the medium.
There is provided as a base, 200 ml of deionized water
and the various stock solutions are added in the amounts
5 stated for 1 liter. For example, if there is to be
employed 10 ml of a stock in the final medium, then 10
ml of the stock are added to the 200 ml of the distilled
water. To ensure the salts stay in solution, stock
solutions are normally added in the order shown in the
10 formulations above. After thoroughly mixing additional
deionized water is added to the mixture to bring it to,
as required 500 ml, and the mixture adjusted in pH to a
value of from about 5.8 to 6.0. The final volume is
brought to 1,000 ml and there is normally added tissue
15 culture Agar, or its equivalent to a level of about 0.8%
by weight. This is to provide some solidity to solution
to reduce flow. The mixture is then autoclaved for about
5 to 20 minutes at a pressure 15-21 lbs/in² to kill any
contaminating organism, and suitably labeled and stored
20 as a sterile medium.

25 Briefly, cotton seeds are sterilized and germinated
on a suitable seed germination medium such as a basal
agar medium in the dark for a time sufficient to produce
seedlings. The normal period of growth is up to about 4
weeks, typically 7 to 14 days.

30 Segments of explants are excised from the seedling.
It is preferred that the explant come from the hypocotyl
or cotyledon. In the alternative, one can equally use
immature embryos obtained from the developing fruits of
greenhouse or field grown cotton plants as the explant.
The explant segments are cultured on a suitable first
callus growth medium, preferably a or full Murashige and
Skoog (MS) nutrient medium containing glucose. Growth
occurs by culturing at a temperature of from about 25 to
35 about 35°C in a light/dark cycle of about 16 hours of

1 light and above 8 hours of dark. Culturing is the
procedure whereby the medium is replaced at periodic
intervals as the nutrients are consumed and continued
for approximately about 3 to about 4 weeks, or until
5 undifferentiated callus are formed. The callus are
transferred to a second callus growth medium, preferably
an MS medium supplemented with naphthaleneacetic acid
(NAA) and sucrose as the carbon source and cultured for
three to four months to produce embryos.

10 The embryos may then be maintained in the second
callus growth medium to maintain an embryo supply or
transferred to a plant germination medium such as Beasley
and Ting's medium preferably containing casein hydrolysate
and source of ammonium cultured for 2 to 3 weeks to
15 produce plantlets.

The plantlets are transferred to soil under high
humidity conditions, then transplanted to larger pots in
a greenhouse and finally transferred to the field for
growth to maturity.

20 The methods briefly described herein have been
successfully employed to induce somatic embryo formation
in cotton of the species Gossypium hirsutum by tissue
and suspension cultures and, ultimately, to obtain mature
plants from hypocotyl and cotyledon derived callus cultures
25 of Acala varieties of Gossypium hirsutum including SJ2,
SJ4, SJ5, B1644, B1810, B2724, GC510 and C1 and non
Acala "picker" Siokra and "stripper" variety FC 2017.
Cultures have been transformed to normal plants with
novel traits or properties.

30 More particularly, the procedure involves first the
sterilizing of the cotton seeds. Suitable sterilization
may be achieved by immersing the seeds in 95% ethanol
for 2 to 3 minutes, rinsing in sterile water one or more

1 times, then soaking the seeds in a 15% solution of sodium
hypochlorite for 15 to 20 minutes, and rinsing several
times with sterile water.

5 The sterilized seeds are then transferred to a
first medium, termed a seed germination medium. A seed
germination medium is one of normal salt content. A
suitable germination medium is a basal agar medium,
including White's medium or half-strength MS medium.
10 (One-half ingredient strength). Germination normally
occurs in the dark over an about 12 to about 14 day
period.

15 Hypocotyl and/or cotyledons are preferably excised
from the germinated seed, subdivided or cut into segments
and cultured on a first callus growth medium such as an
MS medium supplemented with growth substances. The
presently preferred medium is the MS medium supplemented
with about 0.4 mg/l thiamine hydrochloride, about 30 g/l
glucose, about 2 mg/l naphthaleneacetic acid, about 1
mg/l kinetin, a common growth regulator, and about 100
20 mg/l inositol and agar. Thiamine hydrochloride can
generally range in concentration from 0.1 to about 0.5
mg/l, glucose about 20 to about 30 g/l, about 1 to about
10 mg/l naphthaleneacetic acid, about 1 to about 2 mg/l
kinetin and about 50 to about 100 mg/l inositol.

25 The cultures are maintained at a temperature of
about 25 to about 35°C, preferably about 30°C and with a
light/dark cycle of about 16 hours of light and about 8
hours of dark. It is preferred to have a light intensity
of about 2000 to 4000 lux, more preferably about 3000 to
30 4000 lux.

35 The calli formed are periodically subcultured at 3
to 4 week intervals and transferred to a fresh first callus
growth medium. In the culturing of the explants, secre-
tions of phenolic compounds from the explants can occur
as evidenced by darkening of the cultured medium. In

1 this instance, the medium is changed more regularly.
Darkening has been avoided by changing the culture medium
every 10 days. Normally, after three to five medium
changes, phenolic secretions will disappear. When this
5 occurs, the first callus growth medium can be replaced
by fresh callus growth medium containing sucrose or sup-
plemented with sucrose as a carbon source.

10 After 3 to 4 weeks of culture, active calli develop
on the cut surfaces of the explants. The calli are then
transferred to a fresh second callus growth maintenance
medium which is preferably an MS medium combined with
about 1 to about 10 mg/l, preferably about 1 to about 5
mg/l NAA. Cytokinin is employed at a concentration of
15 from 0 to about 1 g/l. A callus growth medium is
characterized as a high salt content medium containing
as much as 10 times more salt than the seed germination
medium. The essential difference between first and
second callus growth medium is the carbon source. Glucose
is used during period of phenolic secretions. Sucrose
20 is used when secretion have stopped. The balance of the
callus growth medium can remain the same or changed.

25 The calli are transferred in regular intervals to a
fresh callus growth medium and, after generally about 5
to 7 passages or until an anthocyanin pigmentation becomes
evident in a portion of the calli, which is followed by
development of a yellowish-white embryogenic callus.

30 The embryogenic callus are then selectively
subcultured and maintained by regular subculturing. The
embryogenic callus contain somatic embryos at various
stages of development. Some may have reached the point
of development that enables growth into small plantlets.
Most, however, require further development. Some may be
advanced to germination. Other may be maintained as a
source of embryos for future use.

1 With reference to FIG. 2, there is illustrated this
stage of development showing calli of Acala cotton 10
with somatic embryos 12 of differing size with some
having emerging leaves 14 and roots 16. FIG. 3 illustrates
5 a somatic embryo isolated at a late globular stage.

With reference to FIG. 4, further development may
be achieved by transferring the somatic embryos to a
third growth medium termed herein an embryo germination
10 medium, a medium rich in nitrogen usually in the form of
ammonia or its equivalent. Suitable media include Beasley
and Ting's medium, preferably supplemented with up to about
500 mg/l casein hydrolysate.

15 Germination occurs from somatic embryos and, within
2 to 3 weeks, a well developed plantlet 18 of up to 6
leaves and good root system is generally formed.

At this stage, the plantlets are transferred to
soil in small clumps and grown in a standard incubator
under conditions of high humidity. Temperature is normally
maintained at about 25 to 30°C (See Fig. 7).

20 After a period of growth, the small plants are
transferred to larger pots in a greenhouse and thereafter
transferred to field and grown to maturity. All the
regenerated plants are preferably self-pollinated either
while growing in the green house or in field conditions
25 and the seeds collected. Seeds are then germinated and
4 to 5 week old seedlings transferred to the field for
progeny row trials and other standard plant breeding
procedures. Practicing the above procedure produces
viable cotton plants from about 35% of the explants in
30 the period of time from about 6 to about 8 months.

Proliferation of Embryogenic Cotton Cells
In Suspension Cultures

As an alternative to allowing the growing embryogenic
calli to be developed into a plant, the callus may be cut

1 into smaller pieces and further developed using suspension
culture techniques.

5 In this procedure, suspension concentration is normally from about 750 to 1000 mg of callus parts to 8 ml. callus growth medium such as the second callus growth medium (MS medium supplemented with NAA), and allowed to grow in suspension. In a preferred embodiment, the suspension of the callus is inserted in T-tubes and placed on a roller drum rotating at about 1.5 rpm under
10 a light regime of about 16 hours of light and about 8 hours of dark. Growth is for about 3 to 4 weeks.

15 After about every 3 to 4 weeks, the suspension is filtered to remove large cell clumps of embryogenic callus depicted in groups in FIG. 5 and as isolated at late globular stages as shown in FIG. 6. The filtrate is returned to a nutrient medium for a 3 to 4 week period of growth. This procedure is repeated over and over with harvesting of large clumps at about 3 to 4 week intervals, at which time the medium is supplanted in whole or in
20 part with fresh callus growth medium. Preferably, about 4 volumes or more of the fresh medium are added to about one volume of residual suspension. It is presently preferred that the filter employed have a mesh size greater than about 600 microns, preferably greater than
25 800 microns, as it has been observed the cell masses of a particle size less than 600 microns will not develop into plants, whereas cell masses greater than 600 microns and preferably greater than 800 microns have undergone sufficient differentiation so as to become embryogenic and capable of developing into viable plants.

30 Suspension cultures can also be initiated by transferring of embryogenic calli to a flask, such as a DeLong or Erlenmeyer flask, containing the liquid embryo growth medium in an amount of about 20 ml of MS and NAA at a
35 concentration of 2.0 mg/l. The flask is placed on a

1 gyrotory shaker and is shaken at about 100-110 strokes
per minute. After 3 to 4 weeks the suspension is suitable
for filtration as described above to remove the large
cell clumps for plant development.

5 More typically, after the third or fourth subcul-
ture, the cell suspension from the "T" tube or De Long
or Erlenmeyer flask is plated onto agar-solidified MS
medium containing NAA (2.0 mg/l) or Beasley & Ting's medium
containing casein hydrolysate (500 mg/l) media and a
10 source of nitrogen. Within 3-4 weeks embryogenic calli
with developing embryos become visible. Likewise, the
larger cell clumps when plated on the above media give
rise to embryogenic clumps with developing embryos.

15 In both suspension growth methods, the MS media is
used to promote and/or sustain embryos whereas the
germination medium is employed for rapid plant development.

20 The seedling explants, if desired, can be transformed.
In this procedure, cotyledon and/or hypocotyl segments of
the sterilized seed can be used. Cotyledons are preferred.

25 The segments are placed in a medium containing an
Agrobacterium vector containing a code (genetic marker)
such as resistance to an antibiotic, such as for instance
kanamycin for a time sufficient for the vector to transfer
the gene to the cells of the explant. Generally, contact
times ranging from 1 minute to 24 hours may be used and
may be accompanied with intermittent or gentle agitation.
The explants are then removed and placed on agar-solidified
callus growth medium such as a MS medium supplemented
with NAA (2 mg/l) and incubated about 15 to 200 hours at
30 25 to 35°C, preferably 30°C, on a 16:8 hour light: dark
regime.

35 After incubation, the explants are transferred to
the same medium supplemented with the antibiotic cefotaxime
preferably in a concentration of 200 mg/l. Cefotaxime
is included to prevent any remaining Agrobacterium from

1 proliferating and overgrowing the plant tissues.
5 Alternatively, the explants can be rinsed with MS medium
supplemented with NAA (2mg/l) and incubated an additional
4 to 28 days before rinsing, then incubating the same
10 medium containing cefotaxime. At the end of 4-5 weeks
of culture on fresh medium, the developing callus, i.e.,
primary callus, is separated from the remainder of the
primary explant tissue and transferred to MS medium
containing NAA (2 mg/l), cefotaxime (200 mg/l) and an
15 antibiotic such as kanamycin sulfate (50 mg/l).
Transformed primary callus, identified by virtue of its
ability to grow in the presence of the antibiotic
(kanamycin), is selected and embryos developed, germinated
20 and plants obtained following the procedure set forth
above.

It is also feasible to achieve transformation of a
cell suspension. Following a normal subculture growth
cycle of 7 to 14 days, usually 7 to 10 days, cells are
allowed to settle leaving a supernatant which is removed.
25 The remaining concentrated suspended cells may be
centrifuged at 4000Xg for 5 minutes and the excess medium
is discarded. The concentrated suspension cultures are
resuspended in the 8 ml of the same medium which contains
the Agrobacterium. The suspension is transferred to "T"
tubes and suitably agitated for incubation.

Following about 2 to 24 hours, preferably 3 to 5
hours, of incubation to allow for bacterial attachment and
DNA transfer, the suspension is removed and allowed to
settle. The supernatant containing the bacteria is
30 discarded and the cells are washed with fresh medium.
The suspension may, if desired, be centrifuged for about
5 minutes and the supernatant removed. In either event,
the cells are resuspended in the same medium and
transferred to a "T" tube or flask and suspension subcul-
35 ture resumed. The object is to minimize the amount of
unattached Agrobacterium vector left in the cell suspension.

1 After about 15 to about 200 hours, typically 15 to
about 72 hours, preferably 18 to 20 hours, the suspension
is filtered to remove large clumps and washed with fresh
5 liquid medium and allowed to settle. The suspension is
resuspended in the fresh liquid medium containing
cefotaxime (200 mg/l) plated on a solidified medium in
Petri dishes.

10 Alternatively, the suspension may be resuspended in
fresh medium containing cefotaxime and allowed to grow
an additional 4 to 28 days prior plating on solidified
medium in Petri dishes. Cell concentration is 1 vol. of
suspension cells plus 3 vol. of medium with cefotaxime.
15 Kanamycin at 10 to 300 mg/l preferably about 20 to 200
mg/l more preferably about 40 to 80 mg/l is included in
the medium for selection of transformed cells expressing
the neomycin phosphotransferase (NPT) gene. Cells and
embryos proliferating in the selective concentration of
kanamycin are further grown as set forth above to mature
20 somatic embryos capable of germinating and regenerating
into whole plants according to the procedures described
herein.

25 Using the above procedure and with reference to
FIG. 9, there is shown variable cell colonies which is
consequence of transformation. There exists cotton
cells 20 exhibiting resistance to the antibiotic kanamycin.
With reference to FIG. 10, transformed calli are shown
developing into somatic embryos on an antibiotic MS
medium. FIG. 11 shows transformed somatic embryos
30 established to have kanamycin resistance and transformed
to have resistance to the herbicide glyphosate. FIG. 12
shows plants from the embryos of FIG. 11. FIG. 13 shows
cells transformed to have resistance to lepidopterous
insects growing on an MS medium and in FIG. 14 trans-
ferred to a Beasley and Ting's medium whereas FIG. 15
35 shows further development of the plantlets of FIG. 14 to
more mature plantlets.

Example 1

5

Regeneration of plants starting
from cotyledon explants

Seeds of Acala cotton variety SJ2 of Gossypium hirsutum were sterilized by contact with 95% alcohol for three minutes, then twice rinsed with sterile water and 10 immersed with a 15% solution of sodium hypochlorite for 15 minutes, then rinsed in sterile water. Sterilized seeds were germinated on a basal agar medium in the dark for approximately 14 days to produce a seedling. The cotyledons of the seedlings were cut into segments of 15 2-4mm² which were transferred aseptically to a callus inducing medium consisting of Murashige and Skoog (MS) major and minor salts supplemented with 0.4 mg/l thiamine-HCl, 30 g/l glucose, 2.0 mg/l naphthaleneacetic acid (NAA), 1 mg/l kinetin, 100 mg/l of m-inositol, and agar (0.8%). The cultures were incubated at about 30°C under 20 conditions of 16 hours light and 8 hours darkness in a Percival incubator with fluorescent lights (cool daylight) providing a light intensity of about 2000-4000 lux.

Calli were formed on the cultured tissue segments 25 within 3 to 4 weeks and were white to gray-greenish in color. The calli formed were subcultured every three to four weeks onto a callus growth medium comprising MS medium containing 100 mg/l m-inositol, 20 g/l sucrose, 2 mg/l naphthaleneacetic acid (NAA) and agar. Somatic 30 embryos formed four to six months after first placing tissue explants on a callus inducing medium. The callus and embryos were maintained on a callus growth medium by subculturing onto fresh callus growth medium every three to four weeks.

1 Somatic embryos which formed on tissue pieces were
2 explanted either to fresh callus growth medium, or to
3 Beasley & Ting's medium (embryo germination medium).
4

5 The somatic plantlets which were formed from somatic
6 embryos were transferred onto Beasley and Ting's medium
7 which contained 1200 mg/l ammonium nitrate and 500 mg/l
8 casein hydrolysate as an organic nitrogen source. The
9 medium was solidified by a solidifying agent (Gelrite)
10 and plantlets were placed in Magenta boxes.
11

12 The somatic embryos developed into plantlets within
13 about three months. The plantlets were rooted with six
14 to eight leaves and about three to four inches tall and
15 were transferred to soil and maintained in an incubator
16 under high humidity for three to four weeks and then
17 transferred to a greenhouse. After hardening, plants
18 were also transferred to open tilled soil.
19

Example 2

20 The procedure of Example 1 was repeated using instead
21 half-strength MS medium in which all medium components
22 have been reduced to one-half the specified concentration.
23 Essentially the same results were obtained.
24

Example 3

25 The procedures of Examples 1 and 2 were repeated
26 except that the explant was the hypocotyl segments. The
27 same results were obtained.
28

Example 4

30 The procedure of Examples 1 and 2 were repeated except
31 that the explant was the immature zygotic embryo.
32 Essentially the same results were obtained.
33

1

Example 5

5 The procedure of Examples 1 and 2 was repeated with
Acala cotton varieties SJ4, SJ5, SJ2C-1, GC510, B1644, B
2724, B1810, the picker variety Siokra and the stripper
variety FC2017. All were successfully regenerated.

Example 6

10 The procedure of Example 1 was repeated to the extent of obtaining callus capable of forming somatic embryos. Pieces of about 750-1000 mg of actively growing embryogenic callus was suspended in 8 ml units of liquid suspension culture medium comprised of MS major and minor salts, supplemented with 0.4 mg/l thiamine HCl, 20 g/l sucrose, 100 mg/l of inositol and naphthaleneacetic acid (2 mg/l) in T-tubes and placed on a roller drum rotating at 1.5 rpm under 16:8 light:dark regime. Light intensity of about 2000-4500 lux was again provided by
15 fluorescent lights (cool daylight).

20

After four weeks, the suspension was filtered through an 840 micron size nylon mesh to remove larger cell clumps. The fraction smaller than 840 microns were allowed to settle, washed once with about 20-25 ml of
25 fresh suspension culture medium. This suspension was transferred to T-tubes (2 ml per tube) and each tube diluted with 6 ml of fresh suspension culture medium. The cultures were maintained by repeating the above procedure at 10-12 day intervals. Namely, the suspension
30 was filtered and only the fraction containing cell aggregates smaller than 840 microns was transferred to fresh suspension culture medium. In all instances, the fraction containing cell clumps larger than 840 microns was placed onto the callus growth medium to obtain mature
35 somatic embryos.

1 The somatic embryos that were formed on callus growth
medium were removed and transferred to embryo germination
medium and using the protocol of Example 1 were germinated,
developed into plantlets and then field grown plants.

5

Example 7

10 The procedure of Example 6 was repeated except that
suspension cultures were formed by transferring 750-1000
mg of embryogenic calli to a DeLong flask containing 15-
20 ml of the MS liquid medium containing 2 mg/l NAA.
The culture containing flask was placed on a gyrotory
shaker and shaken at 100-110 strokes/minute. After
three weeks the suspension was filtered through an 840
15 micron nylon mesh to remove the large cell clumps for
plant growth, as in Example 4. The less than 840 micron
suspension was allowed to settle, washed once in the MS
liquid medium and resuspended in 2 to 5 ml of the MS
liquid medium. The suspension was subcultured by transfer
20 to fresh medium in a DeLong flask containing 1-2 ml of
suspension and 15 ml of fresh MS liquid medium. The
cultures are maintained by repeating this procedure at
seven to ten day intervals. At each subculture only the
less than 840 micron suspension was subcultured and the
25 large clumps (840 microns or greater) were used for plant
growth.

Example 8

30 After three or four subcultures using the suspension
growth procedure of Examples 6 and 7, 1.5 to 2.0 ml of
cell suspension from the T-tube and DeLong flask were in
each instance plated onto agar-solidified MS medium
containing 2 mg/l NAA and Beasley & Ting medium containing
35 500 mg/l casein hydrolysate. Within three to four weeks

1 embryogenic calli with developing embryos became visible.
Again, the 840 micron or greater cell clumps were plated
on the callus growth medium giving rise to embryogenic
clumps with developing embryos which ultimately grew
5 into plants.

COTTON TRANSFORMATION

Example 9

10 Transformation To Form Tumorous-Phenotype
 With Agrobacteria LBA 4434

An Acala cotton suspension culture was subcultured
for three to four months in T-tubes with the medium (MS
15 medium containing 2 mg/l NAA) being changed every seven
to ten days. After any medium change thereafter the
cells can be allowed to settle and harvested for
transformation. The supernatant was removed by pipeting
and cells transformed with the Agrobacterium strain LBA
20 4434. The Agrobacterium strain LBA 4434 is described in
(Hoekema, A. et al. Nature 303: 179-180, 1983, incorporated
herein by reference) contains a Ti plasmid-derived binary
plant transformation system. In such binary systems,
one plasmid contains the T-DNA of a Ti-plasmid, the
25 second plasmid contains the vir-region of a Ti-plasmid.
The two plasmids cooperate to effect plant transformation.
In the strain LBA 4434, the T-DNA plasmid, pAL 1050,
contains T_L of pTiAch5, an octopine Ti-plasmid and the
30 vir-plasmid in strain LBA 4434, pAL4404, contains the
intact virulence regions of pTiAch 5 (Ooms, G. et al.
Plasmid 7:15-29, 1982, incorporated herein by reference).
Strain LBA 4434 is available from Dr. Robert Schilperoort
of the Department of Biochemistry, University of Leiden,
The Netherlands.

1 The transforming Agrobacterium strain was taken
from a glycerol stock, inoculated in a small overnight
culture, from which a 50-ml culture was inoculated the
following day. Agrobacteria was grown on YEB medium
5 containing per liter in water adjusted to pH 7.2 with
NaOH, 5 g beef extract, 1 g yeast extract, 5 g peptone,
5 g sucrose. After autoclaving, 1 ml of 2 M MgCl₂ is
added after which antibiotics, as required to kill other
strains. The absorbance at 600 nm of the 50 ml overnight
10 culture is read, the culture centrifuged and the formed
pellet resuspended in the plant cell growth medium (MS
medium plus NAA at 2 mg/l) to a final absorbance at 600
nm of 0.5.

15 Eight ml of this bacterial suspension of Agrobacterium
LBA 4434 was added to each T-tube containing the suspension
of plant cells after removal of the supernatant liquid.
The T-tube containing the plant and bacteria cells was
20 agitated to resuspend the cells and returned to a roller
drum for three hours to allow the Agrobacteria to attach
to the plant cells. The cells were then allowed to
settle and the residual supernatant removed. A fresh
25 aliquot of growth medium was added to the T-tube and the
suspension allowed to incubate on a roller drum for a
period of 18 to 20 hours in the presence of any residual
Agrobacteria which remained. After this time, the cells
20 were again allowed to settle, the supernatant removed and
the cells washed twice with a solution of growth medium
containing cefotaxime (200 ug/ml). After washing, the
30 cells from each T-tube were resuspended in 10 ml growth
medium containing cefotaxime (200 ug/ml in all cases)
and 1 ml aliquots of the suspension plated on petri dishes.

Infected cells grew on the growth medium to which no
phytohormones were added establishing the tissue had
received the wild-type phytohormone genes in T-DNA. The

1 cells developed tumors, further indicating transformation
of the cultures.

Example 10

Transformation of Cotton To Form a Kanamycin-Resistant

Non-Tumorous Phenotype

5 The suspension culture as obtained in Example 9 was
transformed using an Agrobacteria which contained the T-
DNA containing binary vector pCIB 10. (Rothstein, S.J.
10 et al. Gene 53: 153-161, 1987, incorporated herein by
reference) as well as the pAL 4404 vir-plasmid. The T-
DNA of pCIB 10 contains a chimeric gene composed of the
15 promoter from nopaline synthase, the coding region from
Tn5 encoding the enzyme neomycin phosphotransferase, and
the terminator from nopaline synthase. The Agrobacteria
containing pCIB 10 were grown on YEB medium containing
20 kanamycin (50 ug/ml). Transformation was accomplished
in the same manner as in Example 10 except that the 1 ml
aliquots resulting in cells and Agrobacteria were
immediately plated on selective media containing either
25 kanamycin (50 ug/ml) or G418 (25 ug/ml). Expression of
the nos/neo/nos chimeric gene in transformed plant tissue
allows the selection of this tissue in the presence of
both antibiotics. The existence in two to four weeks of
transformed tissue became apparent on the selection
30 plates. Uninfected tissue as well as added control
tissue showed no signs of growth, turned brown and died.
Transformed tissue grew very well in the presence of both
kanamycin and G418.

35 At this time, tissue pieces which were growing well
were subcultured to fresh selection medium. Somatic
embryos formed on these tissue pieces and were explanted
to fresh non-selective growth media. When the embryos
began to differentiate and germinate, i.e., at the point
where they were beginning to form roots and had two or

1 three leaves, they were transferred to Magenta boxes
containing growth medium described in Example 1. Growth
was allowed to proceed until a plantlet had six to eight
leaves, at which time it was removed from the agar medium.

5 The plantlets were now placed in potting soil,
covered with a beaker to maintain humidity and placed in
a Percival incubator for four to eight weeks. At this
time, the plant was removed from the beaker and transferred
to a greenhouse. The plants grew in the greenhouse,
10 flowered and set seed.

Example 11

15 The procedure of Example 10 was followed, except
that the transforming Agrobacteria used contained the T-
DNA vector DEI PEP10 as well as the pAL4404 vir plasmid.
DEI PEP10, shown in Figure 33, utilizes two T-DNA PstI
cleaved right border sequences from A. Tumefaciens (strain
C-58) which had been further subdivided with BamHI for
20 integration in the plant genome, a passenger maize
phosphoenolpyruvate carboxylase gene (Pepcase gene), and
a chimeric gene (NOS/NPT/TK) capable of expression in
plants and conferring resistance to the antibiotics
kanamycin and G418. This chimeric gene utilizes a nopaline
synthetase promoter, the neomycin phosphotransferase II
25 coding region from Tn5, and the terminator from the
herpes simplex virus thymidine kinase gene. Following
transformation, embryogenic callus and embryos were
obtained by selection on kanamycin (50 mg/l). No resistant
callus was obtained from the control (non-transformed
30 callus) plated on kanamycin at this level (50 mg/l).

Example 12

Transformation of Cotton Suspension Culture Cells

To A Glyphosate-Tolerant Phenotype

35 The procedure of Example 10 was followed, except
that the transforming Agrobacteria used contained the T-

1 DNA vector pPMG85/587 (Fillatti, J. et al., Mol Gen.
Genet. 206: 192-199, 1987, incorporated herein by
reference) as well as the pAL4404 vir plasmid. The
5 plasmid pPMG85/587 carries three chimeric genes capable
of expression in plants. Two genes code for neomycin
phosphotransferase (NPT) which confers resistance to the
antibiotics kanamycin and G418. The third chimeric
10 gene, containing the coding sequence from a mutant aroA
gene of *S. typhimurium*, confers tolerance to the herbicide
glyphosate (Comai, et al., Science 221: 370-371, 1983,
incorporated herein by reference). The Agrobacteria
containing pPMG85/587 were grown on medium containing
15 kanamycin (100 ug/ml). Transformation is accomplished
as detailed in Example 10 except that the suspension is
allowed to grow for 28 days at which time 1 ml aliquots
were plated on medium containing selective antibiotics.
Expression of the NPT chimeric gene in transformed plant
20 tissue allowed selection of this tissue on both
antibiotics. In this instance the selective antibiotic
was kanamycin (50 ug/ml).

In two to four weeks, transformed tissue became
apparent on the selection plates. Plant tissue, individual
embryos and callus were then placed on growth medium
containing the herbicide glyphosate 1mM and transformed
25 tissue continued to grow well. Extraction and analysis
of the proteins of both callus and embryos confirmed the
presence of the product of the glyphosate tolerance gene.

Example 13

30 Transformation of Cotton Suspension Culture Cells
To a Hygromycin-Resistant Non-Tumorous Phenotype

The transformation procedure of Example 10 was
followed except there was used as the transforming
35 Agrobacteria one containing the T-DNA binary vector pCIB

1 715 (Rothstein, S. J. et al. Gene 53: 153-161, 1987) as
well as the vir plasmid. The T-DNA of pCIB 715 contains
a chimeric gene composed of the promoter and terminator
from the cauliflower mosaic virus (CaMV) 35S transcript
5 (Odell et al, Nature 313: 810-812, 1985, incorporated
herein by reference) and the coding sequence for hygromycin
B phosphotransferase (Gritz, L. and J. Davies, Gene 25:
179-188, incorporated herein by reference). Agrobacteria
containing pCIB 715 was grown on YEB containing kanamycin
10 (50 ug/ml).

Transformation was accomplished as detailed in
Example 10 again with the change that the 1 ml aliquots
were plated immediately on medium containing as the
selective antibiotic 50 ug/ml hygromycin. Expression of
15 the chimeric hygromycin gene in transformed plant tissue
allows the selection of this tissue on the medium
containing hygromycin. Transformed tissue was grown in
the manner described in Example 8 on the selection growth
medium establishing transformation had occurred.

20

Example 14

Transformation of Cotton Suspension Culture Cells

To Confer Resistance To Lepidopteran Insects

The procedure of Example 10 was followed except where
25 changes are noted below. Different transforming
Agrobacteria were used. Also, after plant tissue was
selected on an antibiotic for the selection of transformed
material, it was further selected for expression of the
BT gene as defined herein.

30 The Agrobacteria used contained the T-DNA vector
pCIB10 (Rothstein et al, Gene 53:153-161 (198) incorporated
herein by reference into which had been inserted the
following chimeric Bacillus thuringiensis endotoxin
genes ("BT Genes"):

35

1 To prepare the Agrobacterium vector there was fused
the CaMV gene VI promotor and protoxin coding sequences.
* A derivative of phage vector mp19 (Yanish-Perron et al.,
1985) was first constructed. The steps are shown in
5 FIGS. 16 and 17. First, a DNA fragment containing
approximately 155 nucleotides 5' to the protoxin coding
region and the adjacent approximately 1346 nucleotides
of coding sequence are inserted into mp19. Phage mp19
10 ds rf (double-stranded replicative form) DNA was digested
with restriction endonucleases SacI and SmaI and the
approximately 7.2-kbp vector fragment was purified after
electrophoresis through low-gelling temperature agarose
by standard procedures. Plasmid pKU25/4, containing
15 approximately 10 kbP (kilobase pairs) of *Bacillus*
thuringiensis DNA, including the protoxin gene, was
obtained from Dr. J. Nueesch, CIBA-Geigy Ltd., Basle,
Switzerland. The nucleotide sequence of the protoxin
gene present in plasmid pKU25/4 is shown in Formula 1
below. Plasmid pKU25/4 DNA was digested with endonucleases
20 HpaI and SacI, and a 1503 bp fragment containing
nucleotides 2 to 1505 of Formula 1 and purified. This
fragment contains approximately 155 bp of bacteria promotor
sequences and approximately 1346 bp of the start of the
protoxin coding sequence. Approximately 100 ng of each
25 fragment is then mixed, T4 DNA ligase added, and incubated
at 15°C overnight. The resulting mixture was transformed
into *E. coli* strain HB 101, mixed with indicator bacteria
E. coli JM 101 and plated. One phage (mp19/bt) was used
for further construction below.

30 Next, a fragment of DNA containing the CaMV gene VI
promotor, and some of the coding sequences for gene VI,
was inserted into mp19/bt. Phage mp19/bt ds rf DNA is
digested with BamHI, treated with the large fragment of
DNA polymerase to create flush ends and recleaved with
35 endonuclease PstI. The larger vector fragment was purified

1 by electrophoresis as described above. Plasmid pABD1 is
described in Paszkowski et al., EMBO J. 3, 2717-2722,
(1984) incorporated herein by reference. Plasmid pABD1
DNA is digested with PstI and HindIII. The fragment
5 approximately 465 bp long containing the CaMV gene VI
promotor and approximately 75 bp of gene VI coding sequence
was purified. The two fragments were ligated and plated
as described above. One of the resulting recombinant
10 phages, mp19/btca contained the CaMV gene VI promotor
sequences, a portion of the gene VI coding sequence,
approximately 155 bp of *Bacillus thuringiensis* DNA upstream
of the protoxin coding sequence, and approximately 1346
bp of the protoxin coding sequence. To fuse the CaMV
15 promotor sequences precisely to the protoxin coding
sequences, the intervening DNA was deleted using
oligonucleotide-directed mutagensis of mp19/btca DNA.
A DNA oligonucleotide with the sequence (5')
TTCGGATTGTTATCCATGGTGGAGGTCTGA (3) was synthesized by
routine procedures using an Applied Biosystems DNA
20 Synthesizer. This oligonucleotide is complimentary to
those sequences in phage mp19/btca DNA at the 3' end of
the CaMV promotor (nucleotides 5762 to 5778 in Hohn,
Current Topics, in Microbiology and Immunology, 96, 193-
235 (1982) incorporate herein by reference and the
25 beginning of the protoxin coding sequence (nucleotides
156 to 172 in formula I above). The general procedure
for the mutagensis is that described in Zoller and Smith,
Meth, Enzym., 100 468-500 (1983) incorporated herein by
reference. Approximately five micrograms of single-standed
30 phage mp19/btca DNA was mixed with 0.3 mg of phosphorylated
oligonucleotide in a volume of 40 ul. The mixture was
heated to 65°C for 5 min, cooled to 50°C, and slowly
cooled to 4°C. Next, buffer, nucleotide triphosphates,
ATP, T₄ DNA ligase and large fragment of DNA polymerase
35 were added and incubated overnight at 15°C as described

1 [Zoller and Smith Meth. Enzym., 100, 468-500 (1983)]
incorporated herein by reference. After agarose gel
electrophoresis, circular double-stranded DNA was purified
and transfected into *E. coli* strain JM101. The resulting
5 plaques are screened for sequences that hybridize with
32P-labeled oligonucleotide, and phage are analyzed by
DNA restriction endonuclease analysis. Among the resulting
phage clones were ones which have correctly deleted the
unwanted sequences between the CaMV gene VI promotor and
10 the protoxin coding sequence. This phage is called
mp19/btca/del (see FIG. 17).

Next, a plasmid was constructed in which the 3' coding
region of the protoxin gene was fused to CaMV transcription
termination signals. The steps are shown in FIG. 18.
15 First, plasmid pABDI DNA was digested with endonucleases
BamHI and BglII and a 0.5 kbp fragment containing the
CaMV transcription terminator sequences isolated. Next
plasmid pUC19, Yanisch-Perron et al., Gene, 33, 103-119
(1985) incorporated herein by reference was digested
20 with BamHI, mixed with the 0.5 kbp fragment and incubated
with *T*₄ DNA ligase. After transformation of the DNA
into *E. coli* strain HB101, one of the resulting clones,
called plasmid p702, was obtained which has the structure
shown in FIG. 18. Next, plasmid p702 DNA was cleaved
25 with endonucleases SacI and SmaI, and the larger,
approximately 3.2 kbp fragment isolated by gel
electrophoresis. Plasmid pKU25/4 DNA was digested with
endonucleases AhaIII and SacI, and the 2.3-kbp fragment
(nucleotides 1502 to 3773 of Formula 1) containing the
30 3' portion of the protoxin coding sequence (nt 1504 to
3773) was isolated after gel electrophoresis. These two
DNA fragments are mixed, incubated with *T*₄ DNA ligase and
transformed into *E. coli* strain HB101. The resulting
plasmid was p702/bt (FIG. 18).

1 Finally, portions of phage mp19/btca/del ds rf DNA
and plasmid p702/bt were joined to create a plasmid
containing the complete protoxin coding sequence flanked
by CaMV promoter and terminator sequences (see FIG. 18).
5 Phage mp19/btca/del DNA was digested with endonucleases
SacI and SphI, and a fragment of approx. 1.75 kbp is
purified following agarose gel electrophoresis. Similarly,
plasmid p702/bt DNA is digested with endonucleases SacI
and SalI and a fragment of approximately 2.5 kbp is
10 isolated. Finally, plasmid pBR 322 DNA (Bolivar et al.,
Gene, 2, 95-113 (1977) incorporated herein by reference
was digested with SalI and SphI and the larger 4.2-kbp
fragment isolated. All three DNA fragments were mixed
and incubated with T4 DNA ligase and transformed into E.
15 coli strain HB101. The resulting plasmid, PBR322/bt14
is a derivative of PBR322 containing the CaMV gene VI
promoter and translation start signals fused to the
Bacillus thuringiensis crystal protein coding sequence,
followed by CaMV transcription termination signals (shown
20 in FIG. 19).

25 The vector pCIB10 is a Ti-plasmid-derived vector
useful for transfer of the chimeric gene to plants via
Agrobacterium tumefaciens. The vector is derived from
the broad host range plasmid pRK 252, which may be obtained
from Dr. W. Barnes, Washington University, St. Louis,
Mo. The vector also contains a gene for kanamycin
resistance in Agrobacterium, from Tn903, and left and
right T-DNA border sequences from the Ti plasmid pTiT37.
Between the border sequences are the polylinker region
30 from the plasmid pUC18 and a chimeric gene that confers
kanamycin resistance in plants.

35 First, plasmid pRK252 was modified to replace the gene
conferring tetracycline-resistance with one conferring
resistance to kanamycin from the transposon Tn903 [Oka,
et al., J. Mol. Biol., 147, 217-226 (1981) incorporated

1 herein by reference], and was also modified by replacing
the unique EcoRI site in pRK252 with a BglII site (see
FIG. 20 for a summary of these modifications). Plasmid
pRK252 was first digested with endonucleases SalI and
5 SmaI, then treated with the large fragment of DNA
polymerase I to create flush ends, and the large vector
fragment purified by agarose gel electrophoresis. Next,
plasmid p368 was digested with endonuclease BamHI, treated
with the large fragment of DNA polymerase, and an
10 approximately 1050-bp fragment isolated after agarose
gel electrophoresis; this fragment containing the gene
from transposon Tn903 which confers resistance to the
antibiotic kanamycin [Oka et al., J. Mol. Biol., 147,
15 217-226 (1981) incorporated herein by reference]. Both
fragments were then treated with the large fragment of DNA
polymerase to create flush ends. Both fragments are
mixed and incubated with T4 DNA ligase overnight at
15°C. After transformation into E. coli strain HB101
and selection for kanamycin resistant colonies, plasmid
20 pRK252/Tn903 is obtained (see FIG. 19).

Plasmid pRK252/Tn903 was digested at its EcoRI site,
followed by treatment with the large fragment of E. coli
DNA polymerase to create flush ends. This fragment was
added to synthetic BglII restriction site linkers, and
25 incubated overnight with T4 DNA ligase. The resulting
DNA was digested with an excess of BglII restriction
endonuclease and the larger vector fragment purified by
agarose gel electrophoresis. The resulting fragment was
again incubated with T4 DNA ligase to recircularize the
30 fragment via its newly-added BglII cohesive ends.
Following transformation into E. coli strain HB101,
plasmid pRK252/Tn903/BglII is obtained (see FIG. 20).

A derivative of plasmid pBR322 was constructed which
contains the Ti plasmid T-DNA borders, the polylinker
35 region of plasmid pUC19, and the selectable gene for

1 kanamycin resistance in plants (see FIG. 21). Plasmid
pBR325/Eco29 contains the 1.5-kbp EcoRI fragment from
the nopaline Ti plasmid pTiT37. This fragment contains
the T-DNA left border sequence; Yadav et al., Proc.
5 Natl. Acad. Sci. USA, 79, 6322-6326 (1982) incorporated
herein by reference. To replace the EcoRI ends of this
fragment with HindIII ends, plasmid pBR325/Eco29 DNA was
digested with EcoRI, then incubated with nuclease S1,
followed by incubation with the large fragment of DNA
10 polymerase to create flush ends, then mixed with synthetic
HindIII linkers and incubated with T4 DNA ligase. The
resulting DNA was digested with endonucleases ClaI and
an excess of HindIII, and the resulting 1.1-kbp fragment
containing the T-DNA left border purified by gel
15 electrophoresis. Next, the polylinker region of plasmid
pUC19 was isolated by digestion of the plasmid DNA with
endonucleases EcoRI and HindIII and the smaller fragment
(approx. 53 bp) isolated by agarose gel electrophoresis.
Next, plasmid pBR322 was digested with endonucleases
20 EcoRI and ClaI, mixed with the other two isolated
fragments, incubated with T4 DNA ligase and transformed
into *E. coli* strain HB101. The resulting plasmid, pCIB5,
contains the polylinker and T-DNA left border in a
derivative of plasmid pBR322 (see FIG. 21).

25 A plasmid containing the gene for expression of
kanamycin resistance in plants was constructed (see
FIGS. 22 and 23). Plasmid Bin6 obtained from Dr. M.
Bevan, Plant Breeding Institute, Cambridge, UK. This
plasmid is described in the reference by Bevan, Nucl.
30 Acids Res., 12, 8711-8721 (1984) incorporate herein by
reference. Plasmid Bin6 DNA was digested with EcoRI and
HindIII and the fragment approximately 1.5 kbp in size
containing the chimeric neomycin phosphotransferase (NPT)
35 gene is isolated and purified following agarose gel
electrophoresis. This fragment was then mixed with plasmid

1 pUC18 DNA which had been cleaved with endonucleases
EcoRI and HindIII. Following incubation with T4 DNA
5 ligase, the resulting DNA was transformed into *E. coli*
strain HB101. The resulting plasmid is called pUC18/neo.
10 This plasmid DNA containing an unwanted BamHI recognition
sequence between the neomycin phosphotransferase gene
and the terminator sequence for nopaline synthase; see
Bevan, *Nucl. Acids Res.*, 12, 8711-8721 (1984) incorporated
herein by reference. To remove this recognition sequence,
15 plasmid pUC18/neo was digested with endonuclease BamHI,
followed by treatment with the large fragment of DNA
polymerase to create flush ends. The fragment was then
incubated with T4 DNA ligase to recircularize the fragment,
and transformed into *E. coli* strain HB101. The resulting
plasmid, pUC18/neo(Bam) has lost the BamHI recognition
sequence.

20 The T-DNA right border sequence was then added next
to the chimeric NPT gene (see FIG. 24). Plasmid
pBR325/Hind23 contains the 3.4-kbp HindIII fragment of
plasmid pTiT37. This fragment contains the right T-DNA
border sequence; Bevan et al., *Nucl. Acids Res.*, 11,
25 369-385 incorporated herein by reference. Plasmid
pBR325/Hind23 DNA was cleaved with endonucleases SacII
and HindIII, and a 1.0 kbp fragment containing the right
border isolated and purified following agarose gel
electrophoresis. Plasmid pUC18/neo(Bam) DNA was digested
30 with endonucleases SacII and HindIII and the 4.0 kbp
vector fragment isolated by agarose gel electrophoresis.
The two fragments were mixed, incubated with T4 DNA
ligase and transformed into *E. coli* strain HB101. The
resulting plasmid, pCIB4 (shown in FIG. 23), contains
the T-DNA right border and the plant-selectable marker
for kanamycin resistance in a derivative of plasmid pUC18.

35 Next, a plasmid was constructed which contains both
the T-DNA left and right borders, with the plant selectable

1 kanamycin-resistance gene and the polylinker of pUC18
between the borders (see FIG. 28). Plasmid pCIB4 DNA
was digested with endonuclease HindIII, followed by
treatment with the large fragment of DNA polymerase to
5 create flush ends, followed by digestion with endonuclease
EcoRI. The 2.6-kbp fragment containing the chimeric
kanamycin-resistance gene and the right border of T-DNA
was isolated by agarose gel electrophoresis. Plasmid
pCIB5 DNA was digested with endonuclease AatII, treated
10 with T4 DNA polymerase to create flush ends, then cleaved
with endonuclease EcoRI. The larger vector fragment was
purified by agarose gel electrophoresis, mixed with the
pCIB4 fragment, incubated with T4 DNA ligase, and
transformed into *E. coli* strain HB101. The resulting
15 plasmid, pCIB2 (shown in FIG. 24) is a derivative of
plasmid pBR322 containing the desired sequences between
the two T-DNA borders.

The following steps complete construction of the
vector pCIB10, and are shown in FIG. 25. Plasmid pCIB2
20 DNA was digested with endonuclease EcoRV, and synthetic
linkers containing BglII recognition sites are added as
described above. After digestion with an excess of
BglII endonuclease, the approximately 2.6-kbp fragment
was isolated after agarose gel electrophoresis. Plasmid
25 PRK252/Tn903/BglII, described above (see FIG. 20) was
digested with endonuclease BglII and then treated with
phosphatase to prevent recircularization. These two DNA
fragments are mixed, incubated with T4 DNA ligase and
transformed into *E. coli* strain HB101. The resulting
30 plasmid is the completed vector, pCIB10.

Insertion of the chimeric protoxin gene into vector
pCIB10 is by the steps shown in FIG. 26. Plasmid
pBR322/btl4 DNA was digested with endonucleases PvuI and
SalI, and then partially digested with endonuclease
35 BamHI. A BamHI-SalI fragment approx. 4.2 kbp in size,

1 containing the chimeric gene, was isolated following
agarose gel electrophoresis, and mixed with plasmid
pCIB10 DNA which had been digested with endonucleases
BamHI and SalI. After incubation with T4 DNA ligase and
5 transformation into E. Coli strain HB101, plasmid shown
in FIG. 26 and contained the chimeric protoxin gene in
the plasmid vector pCIB10.

In order to transfer plasmid pCIB10/19Sbt from E.
coli HB101 to Agrobacterium, an intermediate E. coli
10 host strain S17-1 was used. This strain, obtainable
from Agrigenetics Research Corp., Boulder, Co. contains
mobilization functions that transfer plasmid pCIB10
directly to Agrobacterium via conjugation, thus avoiding
the necessity to transform naked plasmid DNA directly
15 into Agrobacterium (reference for strain S17-1 is Simon
et al., "Molecular Genetics of the Bacteria-Plant
Interaction", A Puhler, ed, Springer Verlag, Berlin,
pages 98-106, 1983, incorporated herein by reference).
First, plasmid pCIB10/19Sbt DNA is introduced into calcium
20 chloride-treated S17-1 cells. Next, cultures of
transformed S17-1 cells and Agrobacterium tumefaciens
strain LBA4404 [Ooms et al., Gene, 14, 33-50 (1981)
incorporated herein by reference] were mixed and mated
on an N agar (Difco) plate overnight at room temperature.
25 A loopful of the resulting bacteria are streaked onto AB
minimal media; Chilton et al., Proc. Natl. Acad. Sci.
USA, 71, 7347-7351 (1974), incorporated herein by
reference, plated with 50ug/ml kanamycin and incubated
at 28°C. Colonies were restreaked onto the same media,
30 then restreaked onto NB agar plates. Slow-growing colonies
were picked, restreaked onto AB minimal media with
kanamycin and single colonies isolated. This procedure
selects for Agrobacteria containing the pCIB10/19Sbt
plasmid.

1 Construction of a *Bacillus thuringiensis* protoxin
chimeric gene with the CaMV 35S promoter was achieved by
construction of a CaMV 35S Promoter Cassette Plasmid
5 pCIB710 was constructed as shown in FIG. 27. This plasmid
contained CaMV promoter and transcription termination
sequences for the 35S RNA transcript [Covey, S.N.,
Lomonossoff, G.P. and Hull, R., Nucleic Acids Research
10 vol. 9, 6735-6747 (1981) incorporated herein by reference].
A 1149-bp BglII restriction fragment of CaMV DNA in Hohn
et al., Current Topics in Microbiology and Immunology,
15 96, 194-220 and Appendices A to G (1982) incorporated
herein by reference] was isolated from plasmid pLV111
(obtained from Dr. S. Howell Univ. California-San Diego;
alternatively, the fragment can be isolated directly
15 from CaMV DNA) by preparative agarose gel electrophoresis
as described earlier and mixed with BamHI-cleaved plasmid
pUC19 DNA, treated with T4 DNA ligase, and transformed
into *E. coli*. The BamHI restriction site in the resulting
20 plasmid has been destroyed by ligation of the BglII
cohesive ends to the BamHI cohesive ends. The resulting
plasmid, called pUC19/35S, was then used in
oligonucleotide-directed in-vitro mutagensis to insert
25 the BamHI recognition sequence GGATCC immediately
following CaMV nucleotide 7483 in the Hohn reference.
The resulting plasmid, pCIB710, contains the CaMV 35S
promotor region and transcription termination region
30 separated by a BamHI restriction site. DNA sequences
inserted into this BamHI site will be expressed in plants
by the CaMV transcription regulation sequences. pCIB710
does not contain any ATG translation initiation codons
35 between the start of transcription and the BamHI site.

Insertion of the CaMV 35S promoter/Terminator Cassette
into pCIB10 occurred by the steps outlined in FIG. 28.
Plasmids pCIB10 and pCIB710 DNAs were digested with
35 EcoRI and SalI, mixed and ligated. The resulting plasmid,

1 pCIB10/710 has the CaMV 35S promoter/terminator cassette
2 inserted into the plant transformation vector pCIB10.
3 The CaMV 35S sequences are between the T-DNA borders in
4 pCIB10, and thus will be inserted into the plant genome
5 in plant transformation.

6 Insertion of the *Bacillus thuringiensis* protoxin gene
7 into pCIB10/710 occurred by the steps outlined in FIG.
8 29. As a source of the protoxin gene, plasmid pCIB10/19Sbt
9 was digested with BamHI and NcoI, and the 3.6-kb fragment
10 containing the protoxin gene was isolated by preparative
11 gel electrophoresis. The fragment was then mixed with
12 synthetic NcoI-BamHI adapter with the sequence 5'-
13 CATGGCCGGATCCGGC-3', then digested with BamHI. This
14 step creates BamHI cohesive ends at both ends of the
15 protoxin fragment. This fragment was then inserted into
16 BamHI-cleaved pCIB10/710. The resulting plasmid,
17 pCIB10/35Sbt, shown in FIG. 29, contains the protoxin
18 gene between the CaMV 35S promoter and transcription
19 termination sequences.

20 Transfer of the plasmid pCIB10/35Sbt into
21 *Agrobacterium tumefaciens* strain LBA4404 was as described
22 above.

23 Construction of a deleted *Bacillus thuringiensis*
24 protoxin gene containing approximately 725 amino acids,
25 and construction of a chimeric gene containing this
26 deleted gene with the CaMV 35S promoter was made by
27 removing the COOH-terminal portion of the gene by cleaving
28 at the KpnI restriction endonuclease site at position
29 2325 in the sequence shown in Formula 1. Plasmid
30 pCIB10/35Sbt (FIG. 29) was digested with BamHI and KpnI,
31 and the approximately 2.2-kbp BamHI/KpnI fragment
32 containing the deleted protoxin gene isolated by
33 preparative agarose gel electrophoresis. To convert the
34 KpnI site at the 3' end to a BamHI site, the fragment
35 was mixed with a KpnI/BamHI adapter oligonucleotide and

1 ligated. This fragment is then mixed with BamHI-cleaved
pcIB10/710 (FIG. 28).

5 A deleted protoxin gene containing approximately 645 amino acids was made by removing the COOH-terminal portion of the gene by cleaving at the BcII restriction endonuclease site at position 2090 in the sequence shown in Formula 1. Plasmid pcIB10/35Sbt (FIG. 29) was digested with BamHI and BcII, and the approximately 1.9-kbp BamHI/BcII fragment containing the deleted protoxin gene 10 isolated by preparative agarose gel electrophoresis. Since BcII creates a cohesive end compatible with BamHI, no further manipulation is required prior to ligating this fragment into BamHI-cleaved pcIB10/710 (FIG. 28). The resulting plasmid, which has the structure 15 pcIB10/35Sbt(BcII) shown in FIG. 31 was selected on kanamycin.

20 The resulting transformants, designated pcIB10/35Sbt(KpnI) and shown in FIG. 30, contain the deleted protoxin gene of approximately 725 amino acids. These transformants are selected on kanamycin.

25 A deleted protoxin gene was made by introducing a BamHI cleavage site (GGATCC). This is done by cloning the BamHI fragment containing the protoxin sequence from pcIB10/35Sbt into mp18, and using standard oligonucleotide mutagenesis procedures described above. After mutagenesis, double-stranded replicative form DNA is prepared from the M13 clone, which is then digested with BamHI. The approximately 1.9-kbp fragment containing the deleted protoxin gene is inserted into BamHI-cleaved pcIB10/710. 30 The resulting plasmid, which has the structure pcIB10/35Sbt(607) shown in FIG. 32 is selected for on kanamycin.

35 The pcIB10/Sbt 607 was used. Transformation was accomplished as detailed in Example 7 with the change that the 1 ml aliquots were plated immediately on medium

1 containing selective antibiotics. This selection medium
2 contained kanamycin (50 ug/ml) or G418 (25 ug/ml).
3 Expression of the NPT chimeric gene in both transformed
4 plant tissue allows the selection of this tissue on either
5 antibiotic.

10 In 2-4 weeks, transformed tissue became apparent on
the selection plates. Plant material was selected on
kanamycin or G418. Plant tissue (either individual
embryos or callus) was then extracted with buffer and
assayed for expression of the BT gene product by ELISA
15 assay. The conditions of extraction are as follows:
per 100mg of tissue, homogenize in 0.1 ml of extraction
buffer containing 50 mM NaCO₃ (pH9.5), 0.05% Triton,
0.05% Tween, 100mMNaCl, 10mM EDTA, 1mM leupeptine, and
15 1mM PMSF. The leupeptine and PMSF are added immediately
prior to use from 100x stock solutions. The tissue was
ground with a motor driven pestle. After extraction, 2M
Tris pH7 was added to adjust pH to 8.0-8.5 then centrifuged
20 at 12,000 RPM in a Beckman microfuge 12 (10 minutes at
4°C), and the supernatant saved for enzyme linked
immunosorbent assay ("ELISA").

25 ELISA techniques as a general tool is described by
M. F. Clark et al in Methods in Enzymology 118:742-766
(1986), incorporated by reference.

30 An ELISA for the Bt toxin was developed using standard
procedures and used to analyze transgenic plant material
for expression of Bt sequences. For this procedure, an
ELISA plate is pretreated with ethanol and affinity-
purified rabbit anti-Bt antiserum (50 ul) at a
concentration of 3 ug/ml in borate-buffered saline (see
below) is added to the plate. This was allowed to incubate
overnight at 4°C. Antiserum was produced in response to
immunizing rabbits with gradient-purified Bt crystals
[Ang, B.J. & Nickerson, K.W.; Appl. Environ. Microbiol.
35 36: 625-626 (1978)], incorporated herein by reference,

1 solubilized with sodium dodecyl sulfate and washed with
ELISA Wash Buffer (see below). It was then treated for
1 hour at room temperature with Blocking Buffer (see
below) washed with ELISA Wash Buffer. Plant extract was
5 added in an amount to give 50 ug of protein (this is
typically ca. 5 microliters of extract). Leaf extraction
buffer as protein is determined by the Bradford method
[Bradford, M., Anal. Biochem. 72:248 (1976) incorporated
herein by reference] using a commercially available kit
10 obtained from Bio-Rad, Richmond, California. If dilution
of the leaf extract is necessary, ELISA Diluent (see
below)] is used. Allow this to incubate overnight at
4°C. After a wash with ELISA Wash Buffer, 50 ul affinity-
purified goat anti-Bt antiserum is added at a concentration
15 of 3 ug/ml protein in ELISA Diluent. This is allowed to
incubate for 1 hour at 37°C, then washed with ELISA Wash
Buffer. 50 ul rabbit anti-goat antibody bound to alkaline
phosphatase [commercially available from Sigma Chemicals,
St. Louis, Mo.] is diluted 1:500 in ELISA Diluent and
20 allowed to incubate for 1 hour at 37°C, then washed with
ELISA Wash Buffer. 50 microliters substrate [0.6 mg/ml
p-nitrophenyl phosphate in ELISA Substrate Buffer (see
below) are added and incubated for 30 minutes at room
temperature. Reaction is terminated by adding 50
25 microliters of 3 M NaOH. Absorbance is read at 405 nm
in modified ELISA reader [Hewlett Packard, Stanford,
Ca.].

Plant tissue transformed with the pcIB10/35SBt(BclI)
when assayed using this ELISA procedure showed a positive
30 reaction, indicating expression of the Bt gene.

EPBS (ELISA Phosphate Buffered Saline)

10 mM NaPhosphate:	Na ₂ HPO ₄	4.68 grams/4 liters
	NaH ₂ PO ₄ .H ₂ O	0.976 grams/4 liters
35 140 mM NaCl	NaCl	32.7 grams/4 liters

pH should be approximately 7.4

Borate Buffered Saline

100 mM Boric acid

25 mM Na Borate

75 mM NaCl

Adjust pH to 8.4-8.5 with HCl or NaOH as needed.

ELISA Blocking Buffer

In EPBS,

1% BSA

0.02% Na azide

ELISA Wash Buffer

10mM Tris-HCl pH 8.0

0.05% Tween 20

0.02% Na Azide

2.5M TRIS

1 ELISA Diluent

In EPBS:

0.05% Tween 20

1% BSA

5 0.02% Na Azide

ELISA Substrate Buffer

In 500 mls,

48 ml Diethanolamine,

10 24.5 mg MgCl₂;

adjust to pH 9.8 with HCl.

ELISA Substrate

15 15 mg p-nitrophenyl phosphate in 25 ml Substrate Buffer.

15

For bioassays, cell suspensions from antibiotic-resistant cell cultures obtained from transformations with these Agrobacteria were initiated. Suspensions were grown in medium supplemented with G418 (25mg/L), and subcultured into fresh antibiotic-containing medium on 7-10 day intervals. Samples of these cultures were then used in bioassays to test for toxicity to lepidopterous insects. Twenty ml aliquots of these cultures were allowed to settle (cell volume = 3-4ml), and resuspended in medium lacking antibiotics. Suspensions were then allowed to grow for an additional two days in this medium to deplete the cells of any residual antibiotic. Two circles of wet Whatman 2.3 cm filter paper were placed in the bottom of a 3/4 oz portion cup.

20

A layer of transformed suspension culture cells 0.2 cm deep was placed onto the filter paper disk. A newly-hatched Manuca sexta or Heliothis virescens larva was placed into each portion cup. Controls were made up of larvae fed on non-transformed suspension culture cells.

25

Discs were replenished on 2-day intervals or as needed.

-49-

1 Manduca larvae generally require more plant material.
The growth rate and mortality of the larvae feeding on
transformed cells compared with the growth rate of larvae
feeding on untransformed cells was scored after 5 days,
5 and clearly affirmed the toxicity of the BT gene product
in transformed cotton cells.

EXAMPLE 15

10 Heliothis virescens eggs laid on sheets of cheesecloth
are obtained from the Tobacco Insect Control Laboratory
at North Carolina State University, Raleigh, North
Carolina. The cheesecloth sheets are transferred to a
large covered glass beaker and incubated at 29 degrees C
15 with wet paper towels to maintain humidity. The eggs
hatched within three days. As soon as possible after
hatching, the larvae (one larva per cup) are transferred
to covered 3/4 oz. plastic cups. Each cup contains
cotton leaf discs. Larvae are transferred using a fine
20 bristle paint brush.

Leaf discs one centimeter in diameter are punched
from leaves of cotton plants and placed on a circle of
wet filter paper in the cup with the larva. At least 6-
10 leaf discs, representing both young and old leaves,
25 are tested from each plant. Leaf discs are replaced at
two-day intervals, or as necessary to feed the larvae.
Growth rates [size or combined weight of all replica
worms] and mortality of larvae feeding on leaves of
transformed plants are compared with those of larva
30 feeding on untransformed cotton leaves.

Larvae feeding on discs of cotton transformed with
PCIB10/35SB5(BcII) show a decrease in growth rate and
increase in mortality compared with controls.

1 It was observed that a certain number of our
regenerated plants (5-10%) appeared to have acquired
genetically heritable phenotypic variations as a
consequence of the process of regeneration. This variation
5 is known as somaclonal variation. The following examples
illustrate how somaclonal variation as a consequence of
our regeneration procedure has been used to introduce
commercially useful new traits into cotton varieties.

10

EXAMPLE 16

Cotton Regenerants Tolerant to Fungal Pathogens

15 The procedure of Example 1 was followed, and
regenerated cotton plants obtained of the variety SJ5
and SJ4 were hardened and placed in the soil. These
plants were self-pollinated and the seed, representing
the F1 generation, collected.

20 To obtain regenerants (somaclonal variants) more
tolerant to Verticillium, the F1 generation was planted
in a Verticillium infested field for progeny row analysis.
Seed of the varieties SJ4 and SJ5 were planted in the
field as controls. Somaclonal variants more tolerant
25 than the parental varieties to the Verticillium fungus
were identified in a few of the progeny rows (5%) by
assessing overall plant vigor, yield, and the absence of
foliar symptoms associated with the disease. FIG. 33
shows the progeny rows of regenerants planted in a
30 Verticillium infested field. FIG. 34 shows a Verticillium
tolerant somaclonal variant of variety SJ4. This
improvement in tolerance to the fungal pathogen was
found to be genetically stable and passed on to subsequent
generations.

35

-51-

1

EXAMPLE 17

Cotton Regenerants with altered growth habits

The procedure of Example 13 was followed except
5 that, rather than planting in disease-infested soil, the
F1 generation was planted in a cotton breeding nursery.
The overall growth habit of the F1 regenerated progeny
was compared to that of the control varieties. Somaclonal
variants were identified which were more uniform in
10 growth habit and shorter in stature than the parental
variety. One SJ5 regenerant, identified in our trials as
Phy 6, was 20% shorter in stature than the parental
variety. This kind of growth habit is desireable in
cotton grown under narrow row (30" row spacing) cultural
15 conditions. These traits were found to be genetically
stable and passed on to subsequent generations.

EXAMPLE 18

20

Cotton regenerants with improved fiber traits

The procedure of Example 13 was followed except
that the F1 progeny of regenerants were planted in a
cotton breeding nursery and allowed to set fruit. When
the bolls were mature, the cotton was harvested and
25 subjected to an analysis of several fiber quality traits
including length, uniformity, tensile strength, elasticity,
and micronaire. Somaclonal variants were identified
which were improved significantly over the parental
variety in one or more of these traits. Representative
30 data from F2 progeny (cell pollination of the F1) are
included in the following Table 1. Values marked with
an asterisk represent improvements in SJ5 regenerants
which are statistically significant and have been found
to breed true in subsequent generations.

35

1

Table 1

Fiber Properties

	Variety or strain	Length	Uniformity Index	Tensile Strength	Elasticity	Micronaire
5	SJ5	1.13	48.7	24.7	6.8	4.27
10	3SP16	1.27*	51.2	24.6	8.0*	4.10*
15	3SP20	1.28*	53.1*	23.1	7.6*	4.13*
20	5SP10	1.11	53.2*	25.7*	6.2	4.55
25	5SP17	1.18	51.7	26.7*	7.1	4.43

EXAMPLE 19

15

Cotton regenerants with improved yield

20

The procedure of Example 13 was followed except that the F1 progeny of regenerants of the variety SJ4 were planted in replicated yield trials along with nonregenerated controls. One variant, which exhibited a more uniform growth habit and more vigorous growth habit, yielded 4% more cotton than the parental variety in the same trial. The data are given in Table 2 below.

25

Table 2

Variety or Strain	\bar{X} Yield per plot (lb)	\bar{X} Yield lbs/Acre	% Increase
SJ4 Control	28.0	3049	
Phy 4	29.1	3169	4%*

30

*This difference was significant at the 95% confidence level.

A 4% increase in yield would represent a return of almost \$20 per acre to the average cotton grower in

35

-53-

1 California, where over one million acres of cotton are
grown annually.

EXAMPLE 20

5 Cotton Regenerants tolerant to a herbicide. (kanamycin)

Suspension cultures of the cotton variety B1644
were developed according to the method of Example 5.
Suspension cultures were then plated onto an agar medium
as described in Example 6, but supplemented with the
10 herbicide (antibiotic) kanamycin (25mg/l). Most of the
cells in the population died, but a few (1 to 5%) were
tolerant and survived. These were selectively subcultured
onto agar-solidified media supplemented with increasing
concentrations of kanamycin, until the final concentration
15 reached 50mg/l. Embryos were then developed from this
callus, and those resistant embryos were germinated into
kanamycin resistant plants.

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10 20 30 40 50 60
 GTTAAACACCC TGGGTCAAAA ATTGATATTT AGTAAAATTA GTTGCACCTT GTGCATTTT
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 70 80 90 100 110 120
 TCATAAGATG AGTCATATGT TTTAAATTGT AGTAATGAAA AACAGTATTA TATCATAATG
 130 140 150 160 170 180
 AATGGTATC TTAATAAAAG AGATGGAGGT AACTTATGGA TAACAACTCG AACATCAATG
 190 200 210 220 230 240
 AATGCATTCC TTATAATTGT TTAAGTAACC CTGAAAGTAGA AGTATTAGGT GGAGAAAGAA
 250 260 270 280 290 300
 TAGAAACTGG TTACACCCCCA ATCGATATTT CCTTGTCGCT AACGCAATT CTTTGAGTG
 310 320 330 340 350 360
 AATTTGTTCC CGGTGCTGG A TTTGTGTTAG GACTAGTTGA TATAATATGG GGAATTGG
 370 380 390 400 410 420
 GTCCCTCTCA ATGGGACGCA TTTCTGTAC AAATTGAAACA GTTAATTAAC CAAAGAATAG
 430 440 450 460 470 480
 AAGAATTCGC TAGGAAACCAA GCCATTCTA GATTAGAAGG ACTAAGCAAT CTTTATCAAA
 490 500 510 520 530 540
 TTTACGGCAJA ATCTTTAGA GAGTGGGAAG CAGATCCTAC TAATCCAJCA TTAAGGAGAG
 550 560 570 580 590 600
 AGATCCGTAT TCAATTCAT GACAIGAACCA GTGCCCTTAC AACCGCTATT CCTCTTTTG
 610 620 630 640 650 660
 CAGTCAAAAA TTATCAAGTT CCTCTTTAT CAGTATATGT TCAAJCTGCA AATTTACATT
 670 680 690 700 710 720
 TATCAGTTT GAGAGATGTT TCAGTGTGG GACAAAGGTG GGGATTTGAT GCGCGACTA
 730 740 750 760 770 780
 TCAATAGTCG TTATAATGAT TTAACTAGGC TTATTGGCAA CTATAAGAT CATGCTGTAC
 790 800 810 820 830 840
 GCTGGTACAA TACGGGATTA GAGCCTGTAT GGGGACCGGA TTCTAGAGAT TGGATAAGAT
 25
 850 860 870 880 890 900
 ATAATCAATT TAGAAGAGAA TTARCACCAA CTGTATTAGA TATCGTTCT CTATTCGGA
 910 920 930 940 950 960
 ACTATGATAG TAGAACGTAT CCAATTGAA CAGTTCCCA ATTAAACAABA GAAATTATA
 970 980 990 1000 1010 1020
 CAAACCCAGT ATTAGAAAAT TTTGATGGTA GTTTTCGAGG CTCGGCTCAG GGCATAGGAG
 1030 1040 1050 1060 1070 1080
 GAAAGTATTAG GAGTCCACAT TTGATGGATA TACTTAACAG TATAACCACT TATACGGATG
 1090 1100 1110 1120 1130 1140
 CTCATAGAGG AGAATATTAT TGGTCAGGGC ATCAAATAAT GGCTTCTCCT GTAGGGTTT
 1150 1160 1170 1180 1190 1200
 CGGGGCCAGA ATTCACTTT CCGCTATATG GAACTATGGG AAATGCAAGCT CCACAAACAC
 1210 1220 1230 1240 1250 1260
 GAATTGTTGC TCAACTAGGT CABGGCGTGT ATAGAACATT ATCGTCCACT TTATATAGAA
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1270 1280 1290 1300 1310 1320
GACCTTTAA TATAACGATA AATAATCAAC AACTATCTGT TCTTGACGGG ACAGAATTG
1330 1340 1350 1360 1370 1380
CTTATCGAAC CTCCCTCAAAT TTGCGATCCG CTGTATACAG AAAAACCGGA ACGGTAGATT
5 1390 1400 1410 1420 1430 1440
CGCTGGATGA AATACCGCCA CAGAATAACA ACGTGGCACC TAGGCAAGGA TTTAGTCATC
1450 1460 1470 1480 1490 1500
GATTAAGCCA TGTTTCAATG TTTCGTTCAAG CCTTTAGTAA TAGTAGTGTG AGTATAATAA
1510 1520 1530 1540 1550 1560
GAGCTCCTAT GTTCTCTTGG ATACATCGTA GTGCTGAATT TAATAATATA ATTCCCTCAT
10 1570 1580 1590 1600 1610 1620
CACAAATTAC ACAAAATACCT TTAACAAAAAT CTACTAATCT TGGCTCTGGA ACTTCTGTCG
1630 1640 1650 1660 1670 1680
TTAAAGGACC AGGATTTACA GGAGGAGATA TTCTTCAAG AACTTCACCT GGCGAGATT
1690 1700 1710 1720 1730 1740
CAACCTTAAAG AGTAAATATT ACTGCACCAT TATCACAAAG ATATGGGTA AGAATTGCGT
15 1750 1760 1770 1780 1790 1800
ACGCTTCTAC CACAAATTAA CAATTCCATA CATCAATTGA CGGAAAGACCT ATTAATCAGG
1810 1820 1830 1840 1850 1860
GGAATTTTC AGCAACTATG AGTAGTGUGA GTAATTACA GTCCGGARGC TTTAGGACTG
1870 1880 1890 1900 1910 1920
TAGGTTTAC TACTCCGTT AACTTTCAA ATGGATCAAG TGTATTTACG TTAAGTGCCTC
1930 1940 1950 1960 1970 1980
ATGTCTTCAA TTCAGGCAAT GAAGTTATA TAGATEGAAAT TGAATTGTAA CGGGCAGAAG
20 1990 2000 2010 2020 2030 2040
TAACCTTGA GGCAGAATAT GATTTAGAAA GAGCACAAAA GGCGGTGAAT GAGCTGTTA
2050 2060 2070 2080 2090 2100
CTTCTTCCAA TCAAATCGGG TTAAAAACAG ATGTGACGGG TTATCATATT GATCAAGTAT
2110 2120 2130 2140 2150 2160
CCAATTTAGT TGAGTGTTA TCTGATGAAT TTTGTCTGGA TGAAAAAAA GAATTGTCCG
25 2170 2180 2190 2200 2210 2220
AGAAAGTCAA ACATGCGAAG CGACTTAATG ATGAGCGGA TTTACTTCAA GATCCAAACT
2230 2240 2250 2260 2270 2280
TTAGAGGGAT CAATAGAGAA CTAGACCGTG GCTGGAGAGG AAGTACGGAT ATTACCACTC
2290 2300 2310 2320 2330 2340
AAGGAGGGCGA TGACGTATTC AAAGAGAATT ACGTTACGCT ATTGGGTACC TTTGATGAGT
30 2350 2360 2370 2380 2390 2400
GCTATCCAAC GTATTTATAT CAAAAAATAG ATGAGTCGAA ATTAAAGCC TATAACCGTT
2410 2420 2430 2440 2450 2460
ACCAATTAAAG AGGGTATATC GAAGATAGTC AAGACTTACA AATCTATTIA ATTCCGTACA
2470 2480 2490 2500 2510 2520
ATGCCAAACA CGAAACAGTA AATGTGCCAG GTACGGGTTC CTTATGGCCG CTTTCAGCCC

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2530 2540 2550 2560 2570 2580
CAAGTCCTAT CCGAAATGT CCCATCATT CCCATCATTT CTCCTTGGAC ATTGATGTTG
2590 2600 2610 2620 2630 2640
5 GATGTACAGA CTTAAATGAG GACTTAGGTG TATGGGTGAT ATTCAAGATT AAGACGGAAAG
2650 2660 2670 2680 2690 2700
ATGGCCATGC AAGACTAGGA AATCTAGAAAT TTCTCGAAGA GAAACCATTA GTAGGAGAAG
2710 2720 2730 2740 2750 2760
CACTAGCTG TGTGAAAAGA GCGGAGAAAAA AATGGAGAGA CAAACGTGAA AAATTGGAAT
2770 2780 2790 2800 2810 2820
GGGAAACAAA TATTGTTAT AAAGAGGCAA AAGAATCTGT AGATGCTTTA TTTGTAACCT
10 2830 2840 2850 2860 2870 2880
CTCAATATGA TAGATTACAA CGGGATACCA ACATCGCGAT GATTATGCG GCAGATAAAC
2890 2900 2910 2920 2930 2940
GCGTTCATAG CATTGAGAA GCTTATCTGC CTGAECTGTC TGTGATTCG GGTGTCAATG
2950 2960 2970 2980 2990 3000
CGGCTATTT TGAAGAATTA GAGGGCGTA TTTTCACTGC ATTCTCCCTA TATGATGCGA
15 3010 3020 3030 3040 3050 3060
GAAATGTCAT TAAAAATGGT GATTTTAAATA ATGGCTTATE CTGCTGGAAC GTGAAAGGGC
3070 3080 3090 3100 3110 3120
ATGTAGATGT ABAAGAACAA AACAAACCAAC GTTCGGTCCT TGTTGTTCCG GAAATGGGAG
3130 3140 3150 3160 3170 3180
CAGAAGTGTG ACAAGAAGTT CGTGTCTGTC CGGGTCGTGG CTATATCCTT CGTGTACAG
20 3190 3200 3210 3220 3230 3240
CGTACAAGGA GGGATATGGA GAGGGTTGCG TAACCATTCG TGAGATCGAG AACAAATACAG
3250 3260 3270 3280 3290 3300
ACGAACGTGAA GTTTAGCAAC TGTGTAGAAG AGGAAGTATA TCCAAACAAAC ACGGTAACGT
3310 3320 3330 3340 3350 3360
GTAATGATTA TACTGCGACT CAAGAAGAAAT ATBAGGGTAC GTACACTTCT CGTAATCGAG
3370 3380 3390 3400 3410 3420
25 GATATGACGG AGCCTATGAA AGCAATTCTT CTGTACCCAGC TGATTATGCA TCAGCCTATG
3430 3440 3450 3460 3470 3480
AAGAAAAAGC ATATACAGAT GGACGAAGAG ACAAATCCTTG TGAATCTAAC AGAGGGATATG
3490 3500 3510 3520 3530 3540
GGGATTACAC ACCACTACCA GCTGGCTATG TGACAAAAGA ATTAGAGTAC TTCCCAGAAA
3550 3560 3570 3580 3590 3600
30 CCGATAAGGT ATGGATTGAG ATCGGAGAAA CGGAAGGAAC ATTCAACGTG GACAGCGTGG
3610 3620 3630 3640 3650 3660
AATTACTTCT TATGGAGGAA TAATATATGC TTTATAATGT AAGGTGTGCA AATAAAGAAT
3670 3680 3690 3700 3710 3720
GATTACTGAC TTGTATTGAC AGATAAATAA GGAAATTTTT ATATGAATAA AAAACGGGCA
3730 3740 3750 3760 3770 3780
35 TCACTCTAA AAGAATGATG TCCGTTTTT GTATGATTTA ACGAGTGATA TTTAAATGTT

-57-

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	3790	3800	3810	3820	3830	3840
	TTTTTGCAGA AGGCTTTACT TAACGGGGTA CGGCCACATG CCCATCAACT TAAGAATTG					
5	3850	3860	3870	3880	3890	3900
	CACTACCCCC AAGTGTCAAA AAACGTTATT CTTTCTAAAA AGCTAGCTAG AAAGGATGAC					
	3910	3920	3930	3940	3950	3960
	ATTTTTATG AATCTTTCAA TTCAAGATGA ATTACAACTA TTTTCTBAAG AGCTGTATCG					
	3970	3980	3990	4000	4010	4020
	TCATTTAACC CCTTCTCTTT TGGAAGAACT CGCTAAAGAA TTAGGTTTG TAAAAAGAAA					
10	4030	4040	4050	4060	4070	4080
	ACGAAAGTTT TCAGGAAATG AATTAGCTAC CATATGTATC TGGGGCAGTC AACGTACAGC					
	4090	4100	4110	4120	4130	4140
	GAGTGTATTCT CTCGTTGAC TATGCAUTCA ATTACACGCC CGCACAGCAC TCTTATGAGT					
	4150	4160	4170	4180	4190	4200
	CCAGAAGGAC TCAATAAACG CTTTBATAAA AAAGCGGTTG AATTTTGAA ATATATTTT					
15	4210	4220	4230	4240	4250	4260
	TCTGCATTAT GGAAAAGTAA ACTTTGTAAA ACATCAGCCA TTCAAGTGC AGCACTCAGC					
	4270	4280	4290	4300	4310	4320
	TATTTTCAAC GAATCCGTAT TTTAGATGCG ACGATTTCC AAGTACCGAA ACATTTAGCA					
	4330	4340	4350	4360		
	CATGTATATC CTGGGTCAGG TGGTTGTGCA CAAACTGCAG					

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1 WHAT IS CLAIMED IS:

- 1 1. A method for the regeneration of a cotton plant from somatic cells which comprises steps of:
 - 5 a) providing a cotton explant;
 - b) culturing the explant in a first callus growth medium for a period of time sufficient for undifferentiated callus to develop from the explant;
 - c) transferring the callus to a second callus growth medium;
 - 10 d) culturing the callus in the second callus growth medium for a period of time sufficient to allow development of embryogenic callus;
 - e) transferring the embryogenic callus to a plant germination medium; and
 - 15 f) culturing the embryogenic callus on the plant germination medium for a period of time sufficient to develop a plantlet from the embryogenic callus.
- 20 2. The method as claimed in claim 1, in which the cotton seedling is developed by:
 - 25 a) sterilizing the seed in a first sterilizing solution;
 - b) rinsing the seed in sterile water;
 - c) sterilizing the seed in a second sterilizing solution;
 - f) reusing the second sterilization medium from the seed with sterile water;
 - g) transferring the seed to a seed germination medium; and
 - 30 h) growing the seed in the seed germination medium in the dark for a period of time sufficient to produce a seedling; and
 - i) excising the explant from the seedling.

1 3. The method as defined in claim 2 in which the
first sterilizing solution is an aqueous solution
containing about 95% by volume ethanol and the second
sterilizing solution is an aqueous solution containing
5 about 15% by weight sodium hypochlorite.

10 4. The method as claimed in claim 1 wherein the
explant is selected from the group consisting of hypocotyl,
cotyledon and mixtures thereof, and immature zygotic
embryos.

15 5. The method as claimed in claim 2 wherein the
explant is selected from the group consisting of hypocotyl,
cotyledon and mixtures thereof.

20 6. The method as claimed in claim 3 wherein the
explant is selected from the group consisting of hypocotyl,
cotyledon and mixtures thereof.

25 7. A method as claimed in claim 2 in which seed
germination medium is a basal agar medium and growth
prior to explant removal is up to about 4 weeks.

30 8. The method as claimed in claim 1 in which the
embryogenic callus are developed from the explant by
growth in light-dark cycle of about 16 hours of light
and about 8 hours of darkness at a temperature from
about 25 to about 35°C.

35 9. The method as claimed in claim 8 in which the
light intensity during the hours of light is about 2,000
to about 4,000 lux.

10. A method as claimed in claim 8 in which the light

1 intensity during the hours of light is about 3,000 to about
4,000 lux.

5 11. A method as claimed in claim 1 in which callus
are grown in the first callus growth medium containing
glucose during a period of phenolic secretions with
medium changed at least every 10 days.

10 12. The method as claimed in claim 11 wherein the
explant is transferred to a fresh first callus growth
medium at about 3 to about 4 weeks.

15 13. A method as claimed in claim 1 in which the first
callus growth medium is a Murashige and Skoog medium
comprising glucose.

20 14. A method as claimed in claim 1 in which the
second callus growth medium is Murashige and Skoog medium
comprising sucrose and from about 1 to about 10 mg/l
naphthaleneacetic acid.

25 15. A method as claimed in claim 13 in which the
second callus growth medium is Murashige and Skoog medium
comprising sucrose and from about 1 to about 10 mg/l
naphthaleneacetic acid.

30 16. The method as claimed in claim 1 in which the
plant germination in medium is a Beasley and Ting's
medium, rich in a source of nitrogen.

35 17. The method as claimed in claim 15 in which the
step of the plant germination in medium is a Beasley and
Ting's medium, rich in a source of nitrogen.

1 18. The method as claimed in claim 1 further
including the steps of transferring the plantlets to
soil under condition of high humidity for a time sufficient
for the plantlet to mature to enable transfer to a hot
5. house or field for growth to final maturity.

19. A method for the regeneration of a cotton
plant from somatic cells which comprises the steps of:

10 a) providing a cotton explant select from
the group consisting hypocotyl, cotyledon and mixtures
thereof from a cotton seedling and immature embryos;

15 b) culturing the explant in a first callus
growth medium which is a full or half-strength Murashige
and Skoog growth medium supplemented with thiamine
hydrochloride, naphthaleneacetic acid and kinetin and
inositol at a temperature of from about 25 to about 35°C
under a day-light cycle of about 16 hours light at a
light intensity of about 2,000 to about 4,000 lux and
about 8 hours of darkness for a period of time sufficient
20 for undifferentiated callus to form from the explant;

25 c) transferring the callus from the first
callus growth medium to a second callus growth medium
which is a full or half-strength Murashige and Skoog
growth medium comprising sucrose and from about 1 to
about 10 mg/l of naphthaleneacetic acid and culturing
the callus at a temperature from about 25 to 35°C under
a daylight cycle of about 16 hours light at a light
intensity of about 2,000 to about 4,000 lux and about 8
hours dark for a time sufficient to form yellow to white
30 embryogenic callus.

35 d) further subculturing the embryogenic callus
to develop to callus containing somatic embryos;

 e) transferring somatic embryos to an embryo
germination medium rich in a source of nitrogen and

1 growing the embryos to plantlets sufficiently developed
for transfer to soil.

5 20. A method as claimed in claim 19 in which the
first callus growth medium is a Murashige and Skoog
growth medium containing about 0.4 mg/l thiamine
hydrochloride, about 30 g/l sucrose, about 2 mg/l
naphthaleneacetic acid about 1 mg/l kinetin and about 100
mg/l inositol.

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21. A method as claimed in claim 19 in which the
second callus growth medium contains from about 1 to
about 5 mg/l naphthaleneacetic acid and from 0 to about
1 mg/l cytokinin.

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22. A method as claimed in claim 20 in which the
second callus growth medium contains from about 1 to
about 5 mg/l naphthalene acetic acid and from 0 to about
1 mg/l cytokinin.

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23. A method as claimed in claim 19 in which the
embryo germination medium is a Beasley and Ting's medium
containing up to about 500 mg/l casein hydrolysate and up
to about 1200 mg/l ammonium nitrate.

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24. A method as claimed in claim 24 in which the
embryo germination medium contains a source of ammonium.

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25. A method as claimed in claim 22 in which the
embryo germination medium is a Beasley and Ting's medium
containing up to about 500 mg/l casein hydrolysate and
up to about 1200 mg/l ammonium nitrate.

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26. A method as claimed in claim 19 in which the
first callus growth media during a period of phenol

secretion from the callus is changed within about each ten days until phenol secretion stops following which sucrose is included in the callus growth medium.

5 27. A method as claimed in claim 25 in which the
first callus growth media is during a period of phenol
secretion from the callus changed within about each ten
days until phenol secretion stops following which sucrose
is included in the callus growth medium.

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28. A method for the regeneration of cotton plants from somatic cells, comprising the steps of:

(a) sterilizing at least one cotton seed in a solution containing 95% by volume ethanol for a period of approximately 2-3 minutes;

- (b) rinsing the seed in sterile water;
- (c) soaking the seed in a solution of sodium hypochlorite containing about 15% by weight sodium hypochlorite for a period of from about 15 to about 20 minutes;

(d) rinsing the seed in sterile water;
(e) germinating the seed in a dark environment on modified basal agar medium of Whites or half strength Murashige and Skoog medium for a period up to about fourteen days to produce a seedling;

(f) excising segments selected from the hypocotyl, cotyledon or mixtures thereof from the seedling;

(g) culturing the excised segments on a Murashiga-Skoog medium supplemented with about 0.4 mg/l thiamine hydrochloride, about 30 g/l glucose, about 2 mg/l napthaleneacetic acid, about 1 mg/l kinetin and about 100 mg/l inositol for a period of approximately three to four weeks in an environment of 30°C under a light-dark cycle of 16 hours of light and about 8 hours

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1 of dark, at about 3,000 to 4,000 lux light intensity during
the hours of light, to produce callus;

5 (h) transferring the callus onto Murashige and
Skoog medium comprising sucrose and about 2 mg/liter
naphthaleneacetic acid and about 1 mg/liter cytokinin;

(i) culturing the callus over a period of
about three to four months to produce embryos;

10 (j) transferring the embryos to Beasley &
Ting's medium comprising about 500 mg/liter casein
hydrolysate, and a source of nitrogen and culturing the
embryos for a period of about 2 to about 3 weeks, to
produce plantlets; and

15 (k) transferring the plantlets to soil and
incubating the plantlets in high humidity from plants.

27. The method as defined in claim 28 including
the steps of:

20 (a) self-pollinating the plants so as to
produce seeds; and

(b) germinating the seeds to produce seedlings.

30. A method for the regeneration of a cotton
plant from somatic cells which comprises the steps of:

25 a) culturing a cotton explant by tissue culture
on a callus growth medium for a period of time sufficient
to develop embryogenic callus;

b) subdividing and suspending embryogenic callus
in a second callus growth medium and growing said callus
to form embryogenic clumps of at least 600 microns in size;

30 c) filtering out embryogenic clumps of a size
greater than 600 microns;

d) growing the embryogenic clumps of a size greater
than 600 microns in a plant germination medium for a
period of time sufficient to develop plantlets from the
35 clumps.

-65-

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31. A method as claimed in claim 30 in which the
clump of a size less than 600 microns are resuspended in
fresh second callus growth medium for further growth of
5 embryogenic clumps.

32. A method as claimed in claim 30 in which the
explant is obtained by:

- 10 a) sterilizing the seed in a first-sterilized solution;
- b) rinsing the seed with sterile water;
- c) sterilizing the seed in a second sterilizing solution;
- 15 d) rinsing the second sterilization medium from the seed with sterile water;
- e) transferring the sterilized seed to a seed germination medium; and
- 20 f) culturing the seed in the seed germination medium in the dark for a period of time sufficient to form cotton seedling; and
- g) excising the explant from the cotton seedling.

33. The method as claimed in claim 31 in which the
25 explant is obtained by:

- 30 a) sterilizing the seed in a first sterilizing solution;
- b) rinsing the seed with sterile water;
- c) sterilizing the seed in a second sterilizing solution;
- d) rinsing the second sterilization medium from the seed with water;
- e) transferring the sterilized seed to a seed germination medium; and

1 f) culturing the seed in the seed germination
medium in the dark for a period of time sufficient to
form cotton seedling; and
5 g) excising the explant from the cotton
seedling.

10 34. The method of claim 33 in which the first
sterilizing solution is an aqueous solution containing
about 95% by volume ethanol; and the second sterilizing
solution is an aqueous solution containing about 15% by
weight sodium hypochlorite.

15 35. The method as claimed in claim 34, wherein the
explant includes at least a portion of seedling parts
selected from the hypocotyl, cotyledon and the mixtures
thereof.

20 36. A method as claimed in claim 35 in which seed
germination medium is a basic agar medium and growth
prior to explant removal is for a period of up to about
14 days.

25 37. A method as claimed in claim 30 in which clumps
greater than about 800 microns are removed from suspension
for plant growth.

30 38. A method as claimed in claim 30 in which the
suspension culture at the beginning of growth contains from
750 to about 1000 mg of callus parts per 8 ml second
embryo growth medium.

35 39. A method as claimed in claim 31 in which the
suspension culture at the beginning of growth is from
750 to about 1000 mg of callus parts per 8 ml second
embryo growth medium.

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40. The method as claimed in claim 30 in which the embryogenic callus are grown in the suspension in light-dark cycle of about 16 hours of light and about 8 hours 5 of darkness at a temperature from about 25 to about 35°C.

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41. The method as claimed in claim 31 in which the embryogenic callus are grown in the suspension in light-dark cycle of about 16 hours of light and about 8 hours of darkness at a temperature from about 25 to about 35°C.

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42. The method as claimed in claim 40 in which the light intensity during the hours of light is about 2,000 to about 4,000 lux.

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43. A method as claimed in claim 41 in which the light intensity during the hours of light is about 3,000 to about 4,000 lux.

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44. A method as claimed in claim 30 in which the second callus growth medium is Murashige and Skoog medium containing naphthaleneacetic acid.

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45. A method as claimed in claim 31 in which the second callus growth medium is Murashige and Skoog medium containing naphthaleneacetic acid.

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46. The method as claimed in claim 30 in which the step of the plant germination in medium is a Beasley and Ting's medium, rich in a source of nitrogen.

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47. The method as claimed in claim 30 in which the step of the plant germination in medium is a Beasley and Ting's medium, comprising casein hydrolysate and a source of ammonium.

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48. The method as claimed in claim 30 further including the steps of:

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a) transferring the plantlets to soil under condition of high humidity for a time sufficient to mature to enable transfer to a hot house then field for growth to final maturity.

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49. A method for the regeneration of a cotton plant from somatic cells which comprises the steps of:

a) providing an explant selected from the group consisting of hypocotyl, cotyledon and mixtures thereof derived from a cotton seedling and immature embryos;

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b) culturing the explant in a first callus growth medium which is a Murashige and Skoog growth medium supplemented with thiamine hydrochloride, glucose, naphthaleneacetic, kinetin and inositol, at a temperature of from about 25 to about 35°C under a daylight cycle of about 16 hours light at a light intensity of about 2,000 to about 4,000 lux and about 8 hours of darkness for a period of time sufficient for undifferentiated callus to form from the explant;

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c) transferring the callus from the first callus growth medium to a second callus growth medium which is Murashige and Skoog growth medium containing sucrose and from about 1 to about 10 mg/l of naphthaleneacetic acid and culturing the callus at a temperature from about 25 to 35°C under a daylight cycle of about 16 hours light at a light intensity of about 2,000 to about 4,000 lux and about 8 hours dark for a time sufficient to develop embryogenic callus;

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d) suspending parts of the embryogenic callus in fresh second callus growth medium at a concentration from about 750 about 100 mg of callus parts per 8 ml second

-69-

1 embryo growth medium and allowing the embryo to grow for
a time sufficient to develop embryogenic clumps of a
size greater than about 800 microns;

5 e) separating the embryogenic clumps greater than
about 800 microns from clumps less than about 800 microns;

f) transferring the embryogenic clumps of a size
greater than 800 microns to a germination medium rich in
a source of nitrogen and growing the embryos to plantlets.

10 50. A method as claimed in claim 49 in which the
embryogenic clumps less than 800 microns are resuspended
in fresh second callus growth medium as per step d) and
steps e) and f) repeated.

15 51. A method as claimed in claim 49 in which the
first callus growth medium is a Murashige and Skoog
growth medium containing about 0.4 mg/l thiamine
hydrochloride, about 30 g/l glucose, about 2 mg/l
naphthaleneacetic acid, about 1 mg/l kinetin and about
20 100 mg/l inositol.

25 52. A method as claimed in claim 49 in which the
second embryo growth media comprises sucrose and from 1
to about 5 mg/l naphthaleneacetic acid and from 0 to about
1 mg/l cytokinin.

30 53. A method as claimed in claim 50 in which the
second embryo growth medium contains from 1 to about 5 mg/l
naphthaleneacetic acid and from 0 to about 1 mg/l
cytokinin.

35 54. A method as claimed in claim 51 in which the
second embryo grow media contains from 1 to about 5 mg/l
naphthaleneacetic acid and from 0 to about 1 mg/l
cytokinin.

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55. A method as claimed in claim 49 in which the embryo germination medium is a Beasley and Ting's medium containing up to about 500 mg/l casein hydrolysate.

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1 56. A method as claimed in claim 50 in which the
embryo germination medium is a Beasley and Ting's medium
containing up to about 500 mg/l casein hydrolysate.

5 57. A method as claimed in claim 55 in which the
embryo germination medium is a Beasley and Ting's medium
containing up to about 500 mg/l casein hydrolysate.

10 58. A method for transforming cotton which comprises:
a) contacting a cotton explant with a
Agrobacterium vector containing an expressible gene code
foreign to cotton for a time sufficient to transfer the
gene to the cells of explant;
b) incubating the explant in a callus growth
15 medium for about 15 to about 200 hours at a temperature
from about 25 to about 35°C under a cycle of 16 hours
light and 8 hours dark.
c) contacting the incubated explants with a
callus growth media containing an antibiotic which is
20 toxic to the Agrobacterium for a time sufficient to kill
the Agrobacterium;
d) culturing the explant free of the
Agrobacterium in the callus growth media; and
e) selecting transformed callus from
25 untransformed callus.

30 59. A method claimed in claim 58 in which the
supplemented callus growth medium is a Murashige and
Skoog medium supplemented with about 1 to about 10 mg/l
naphthaleneacetic acid.

35 60. A method as claimed in claim 58 in which the
antibiotic toxic to the Agrobacterium is cefotaxime.

1 61. A method for transforming cotton which
comprises;

5 a) contacting for a period of from about 1
minute to about 24 hours, segments of cotton seedling
explant selected from hypocotyl, cotyledon and mixtures
thereof with an Agrobacterium vector containing a gene code
which includes resistance to an antibiotic;

10 b) transferring the explants to a callus growth
medium which is a Murashige and Skoog medium supplemented
with about 1 to about 10 mg/l naphthaleneacetic acid for
a period of from about 15 to about 200 hours at a
temperature of from about 25 to about 35°C under a cycle
of about 16 hours light and 8 hours dark to develop callus
from the explants;

15 c) transferring the callus to a fresh callus
growth medium containing cefotaxime in a concentration
sufficient to kill off Agrobacterium;

20 d) culturing the callus on fresh first callus
growth medium; and

25 e) contacting callus with fresh callus growth
medium additionally containing cefotaxime to kill off
residual Agrobacterium and an antibiotic to which the
transformed callus is insensitive to select callus
resistant to the antibiotic.

30 62. A method as claimed in claim 61 in which the
transformed callus prior to contact with the callus growth
medium containing cefotaxime is rinsed in callus growth
medium free of cefotaxime.

35 63. A method as claimed in claim 61 in which the
antibiotic is kanamycin.

35 64. A method as claimed in claim 62 in which the
antibiotic is kanamycin.

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65. A method of producing transformed embryogenic cotton callus from cotton cells undergoing suspension culture in a callus growth medium which comprises after 5 a suspension subculture growth cycle;

- a) recovering cells and any formed embryogenic callus from the callus growth medium;
- b) resuspending the recovered cells and callus in a callus growth medium containing an 10 Agrobacterium vector including an expressible gene sequence foreign to cotton while maintaining suspension growth conditions for period of time sufficient to transform the cotton cells;
- c) recovering the suspended cells and callus 15 from the callus growth medium containing the Agrobacterium;
- d) treating the transformed cells and callus with an antibiotic which is toxic to the Agrobacterium to kill off the Agrobacterium;
- e) selecting the transformed cotton cells 20 and callus from untransformed cotton cells and embryogenic callus cells; and
- f) filtering a suspension of the cells to remove embryogenic callus greater than about 600 microns.

25 66. A method as claimed in claim 65 in which the transformed cells and callus are treated with the antibiotic prior to filtering the suspension.

30 67. A method as claimed in claim 65 in which the transformed cells and callus are selected prior to filtration of the suspension.

35 68. A method as claimed in claim 66 in which the transformed cells and callus are selected prior to filtration of the suspension.

1 69. A method as claimed in claim 65 in which the
transformed cells and embryogenic callus are separately
treated with the antibiotic following filtration of the
suspension.

5

70. A method as claimed in claim 65 in which the
transformed cells and embryogenic callus are selected
following filtration of the suspension.

10

71. A method as claimed in claim 69 in which the
transformed cells and embryogenic callus are selected
following filtration of the suspension.

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72. A method as claimed in claim 65 in which the
antibiotic toxic to the Agrobacterium is cefotaxime.

73. A method as claimed in claim 66 in which the
antibiotic toxic to the Agrobacterium is cefotaxime.

20

74. A method as claimed in claim 69 in which the
antibiotic toxic to the Agrobacterium is cefotaxime.

25

75. A method of producing embryogenic cotton callus
from cotton cells undergoing suspension culture in a
callus growth medium which comprises after a suspension
subculture growth cycle of from about 7 to about 14 days;

a) recovering cells from the callus growth
medium;

30

b) resuspending the cells in a callus growth
medium containing an Agrobacterium vector including an
expressible gene sequence foreign to cotton while
maintaining suspension growth conditions for period of
time sufficient to transform the suspended cells;

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c) recovering the cells from the callus
growth medium containing the Agrobacterium;

76. A method as claimed in claim 75 in which the antibiotic is kanamycin.

15

77. A method of produced transformed cotton callus from cotton cells undergoing suspension culture in a callus growth medium which comprises after a suspension subculture growth cycle of from about 7 to about 14 days;

20 a) recovering cells from the callus growth medium;

25 b) resuspending the cells in a callus growth medium containing an Agrobacterium vector including an antibiotic resistant gene sequence while maintaining suspension growth conditions for period of time sufficient to transform the suspended cells;

30 c) recovering the cells from the callus growth medium containing the Agrobacterium;

d) washing the cells in fresh callus growth medium free of the Agrobacterium;

e) further subculturing the cells in fresh callus growth medium free of the Agrobacterium;

f) filtering the suspension to remove embryogenic callus greater than about 600 microns;

78. A method as claimed in claim 77 in which the antibiotic is kanamycin.

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79. Cotton plants transformed to have resistance to antibiotics normally inhibitory to cotton plant cell growth.

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80. Cotton plant regenerants exhibiting increased tolerance to *Verticillium* wilt.

81. Cotton regenerants exhibiting improved fiber quality traits of at least greater fiber length, tensile strength, elasticity or lower micronaire as compared to the parental variety.

82. Cotton regenerants exhibiting herbicide tolerance.

25

83. Cotton regenerants exhibiting increased yield as compared to the parental variety.

84. Cotton regenerants exhibiting increased tolerance to fungal pathogens.

85. A vector for conferring antibiotic resistance to a cotton plant which comprises two T-DNA right border sequences from A. Tumefaciens capable of integration with the plant genome flanking a chimeric gene capable

1 of expression in cotton and of conferring resistance to
the antibiotics Kanamycin and G418.

5 86. A vector as claimed in claim 85 in which the
chimeric gene comprises in sequence a napoline synthetase
promoter, a neomycin phosphotransferase II coding region
from Tn5 and the terminator from the herpes simplex
virus thymidine kinase gene.

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Fig. 1

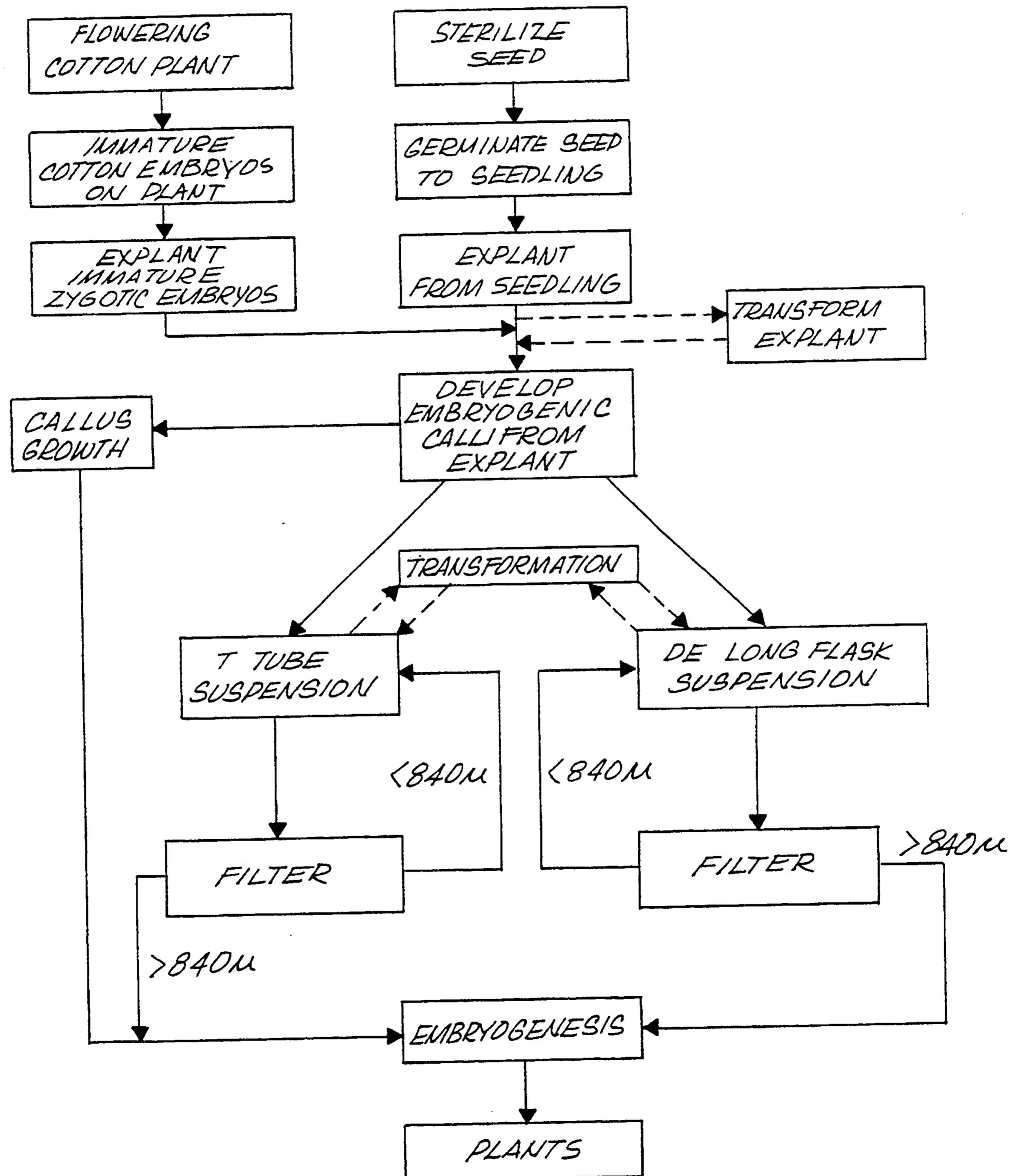
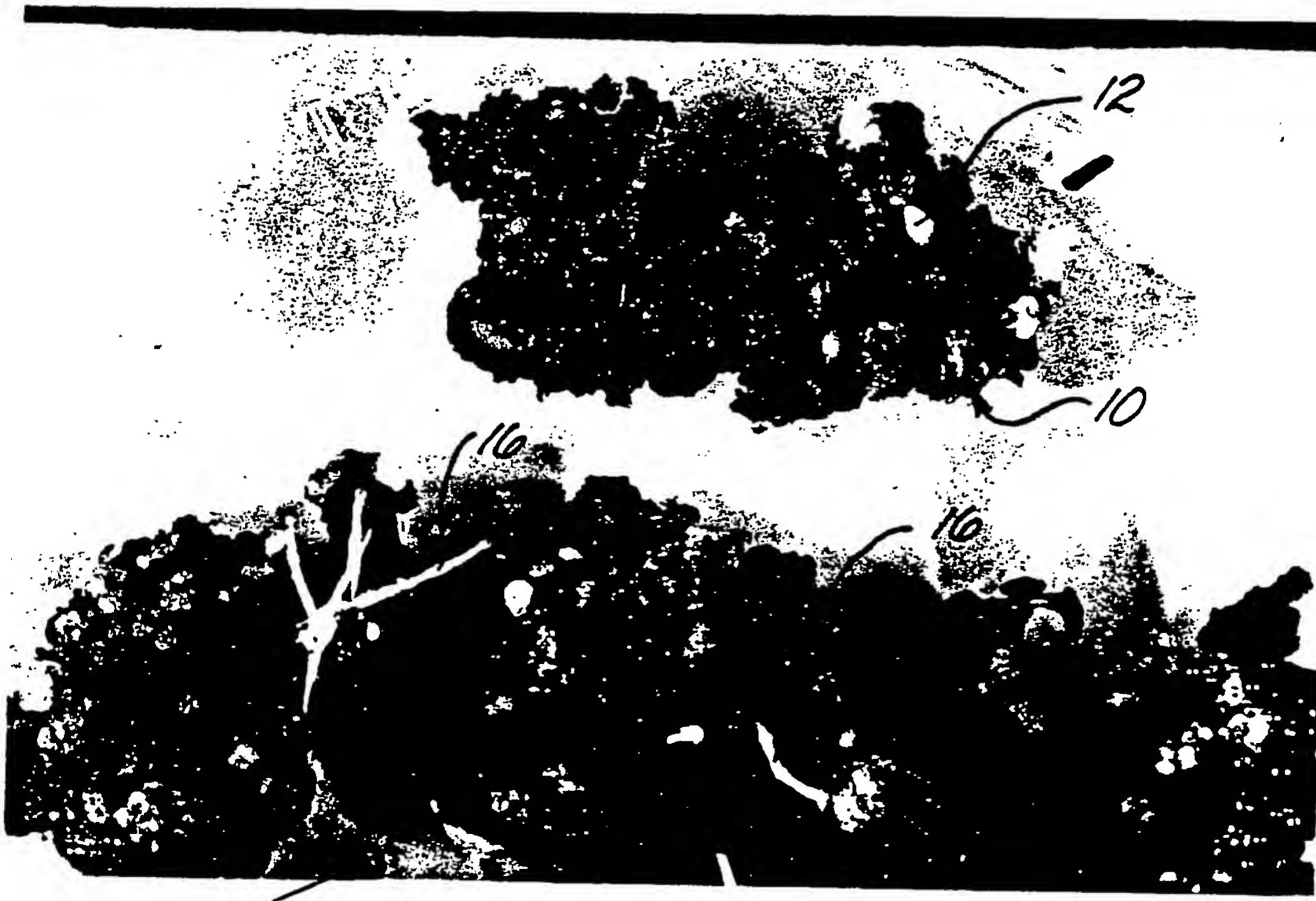


Fig. 2

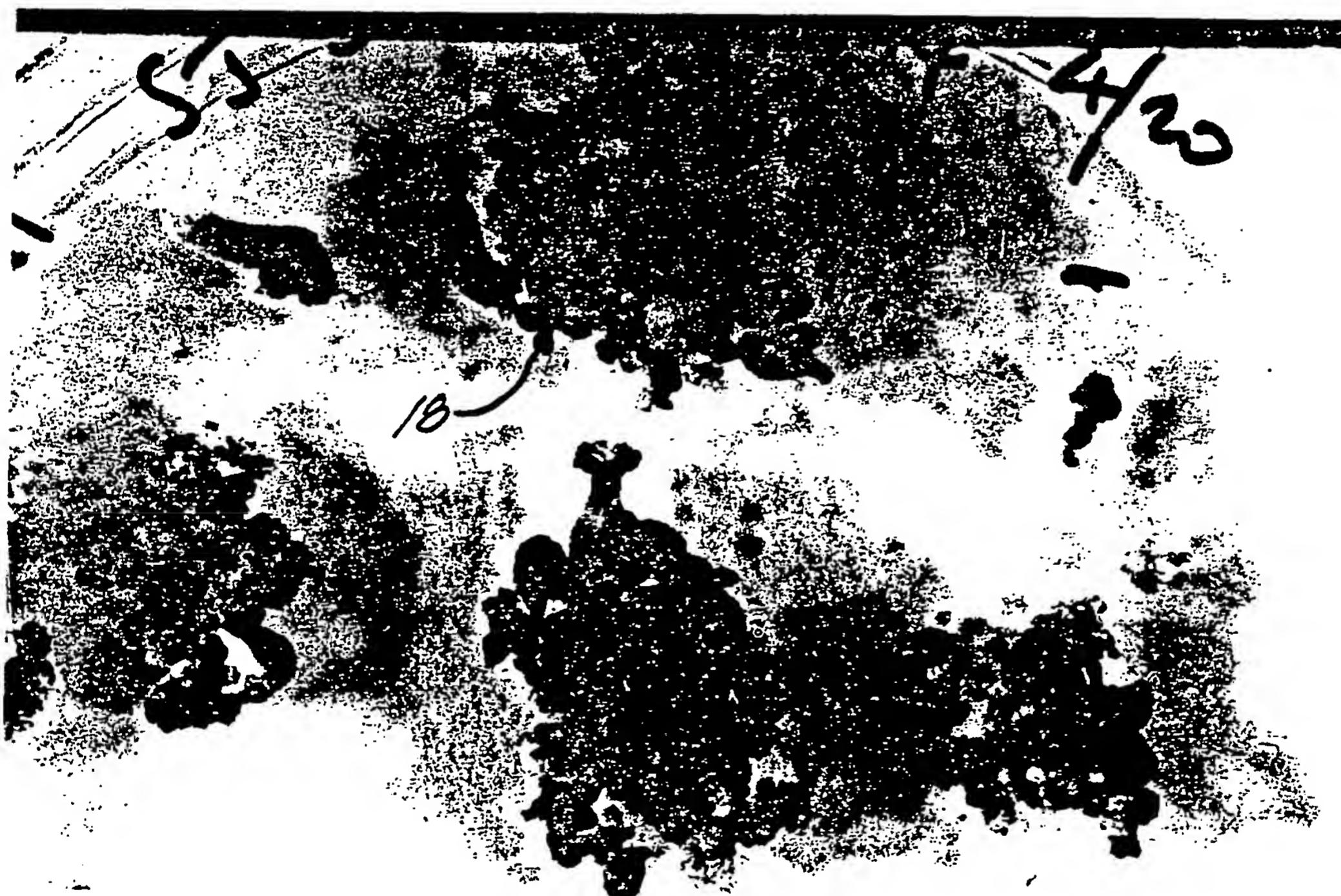


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Fig. 3



Fig. 4

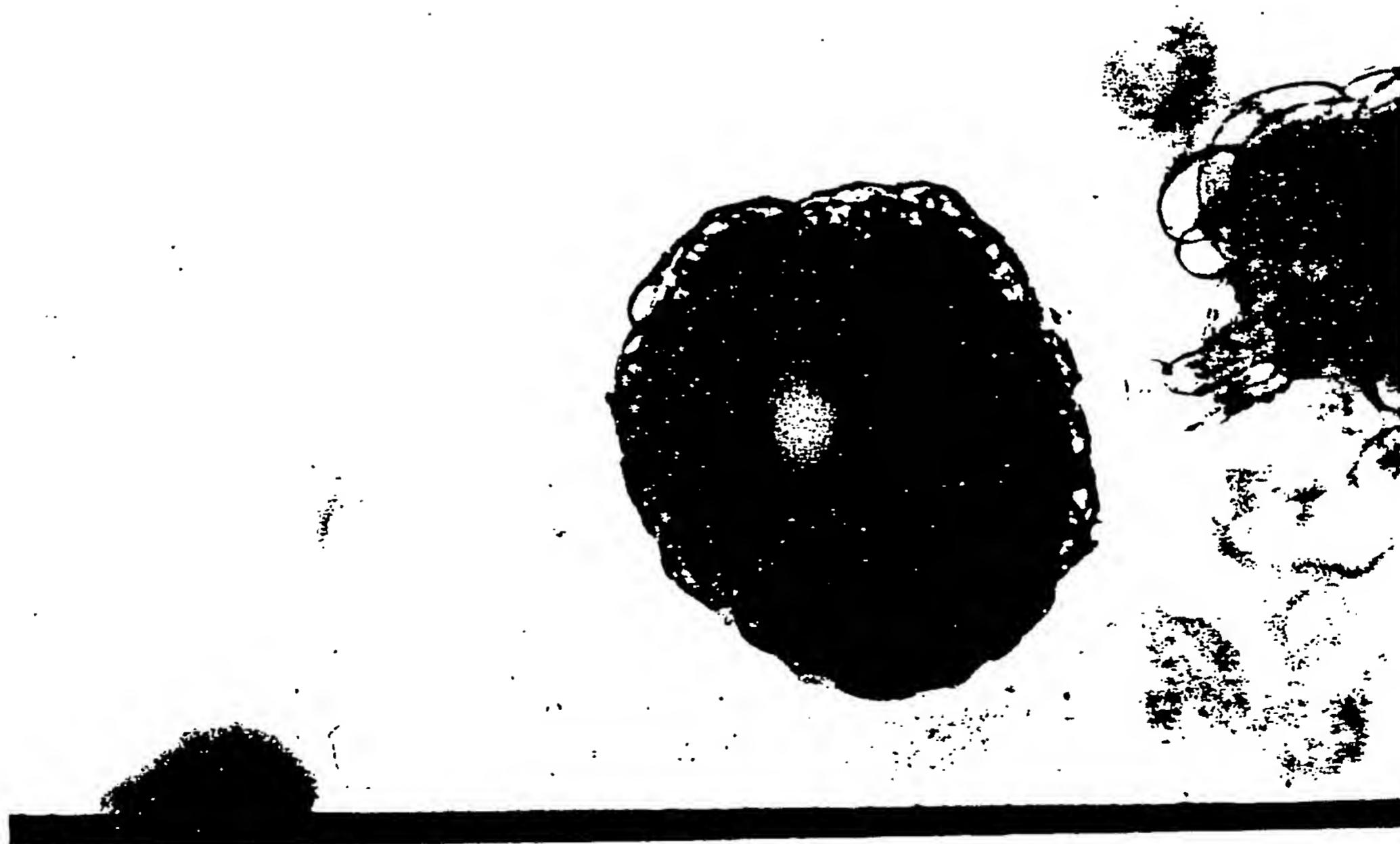


5 / 29

Fig. 5



Fig. 6



6/29

Fig. 7



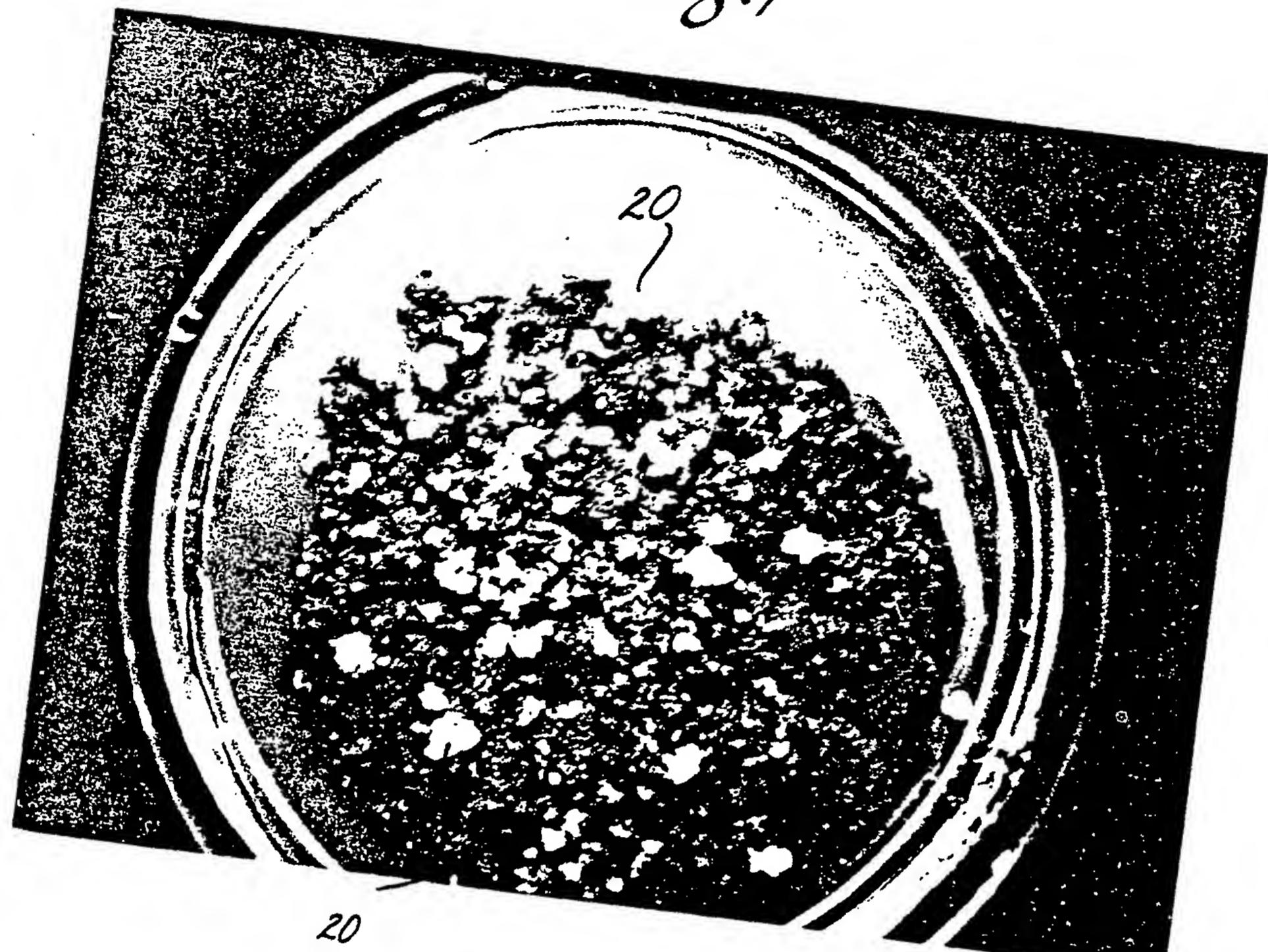
16

16

Fig. 8



7/29
Fig. 9



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Fig. 10



8/29
Fig. 11

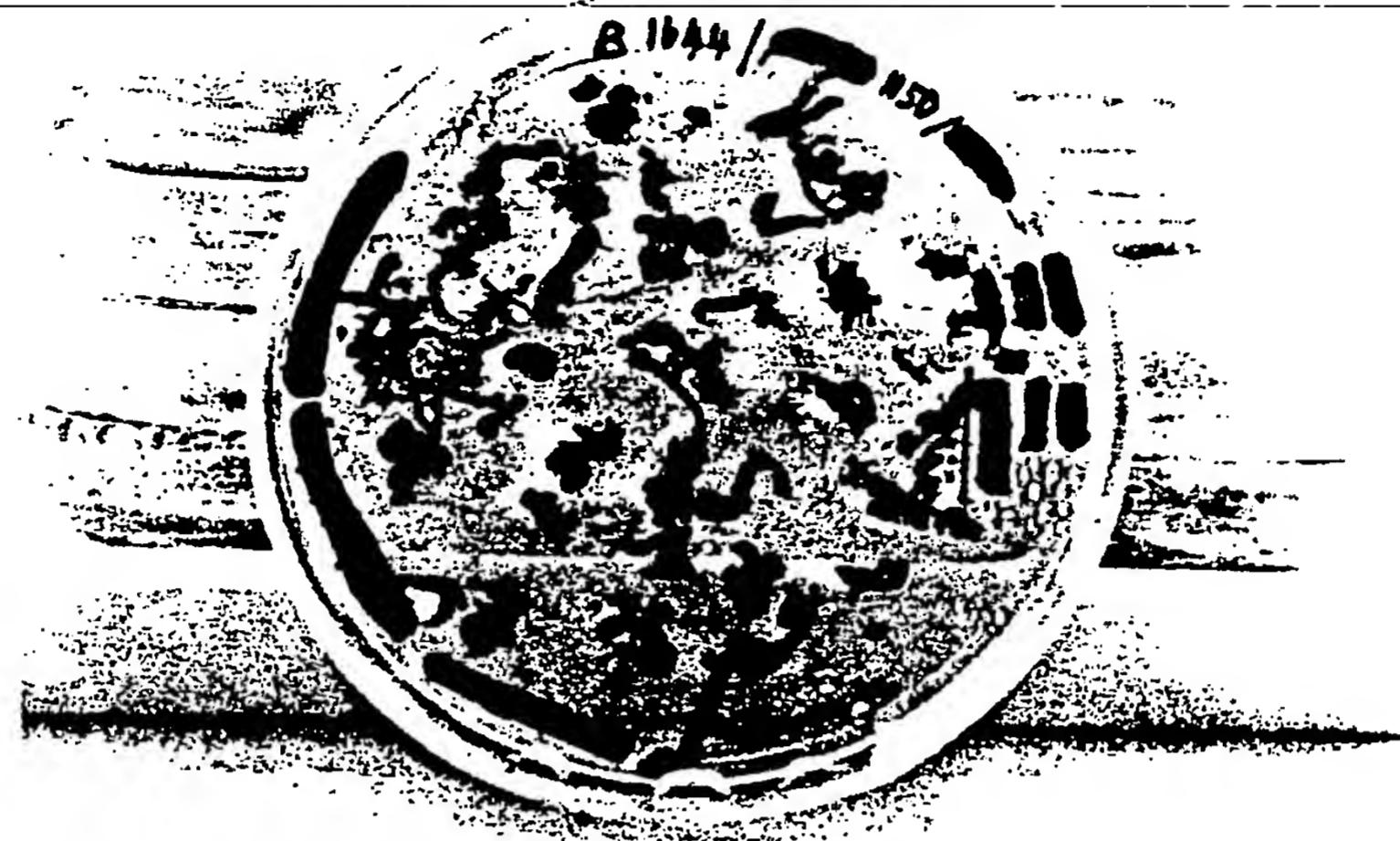


Fig. 12

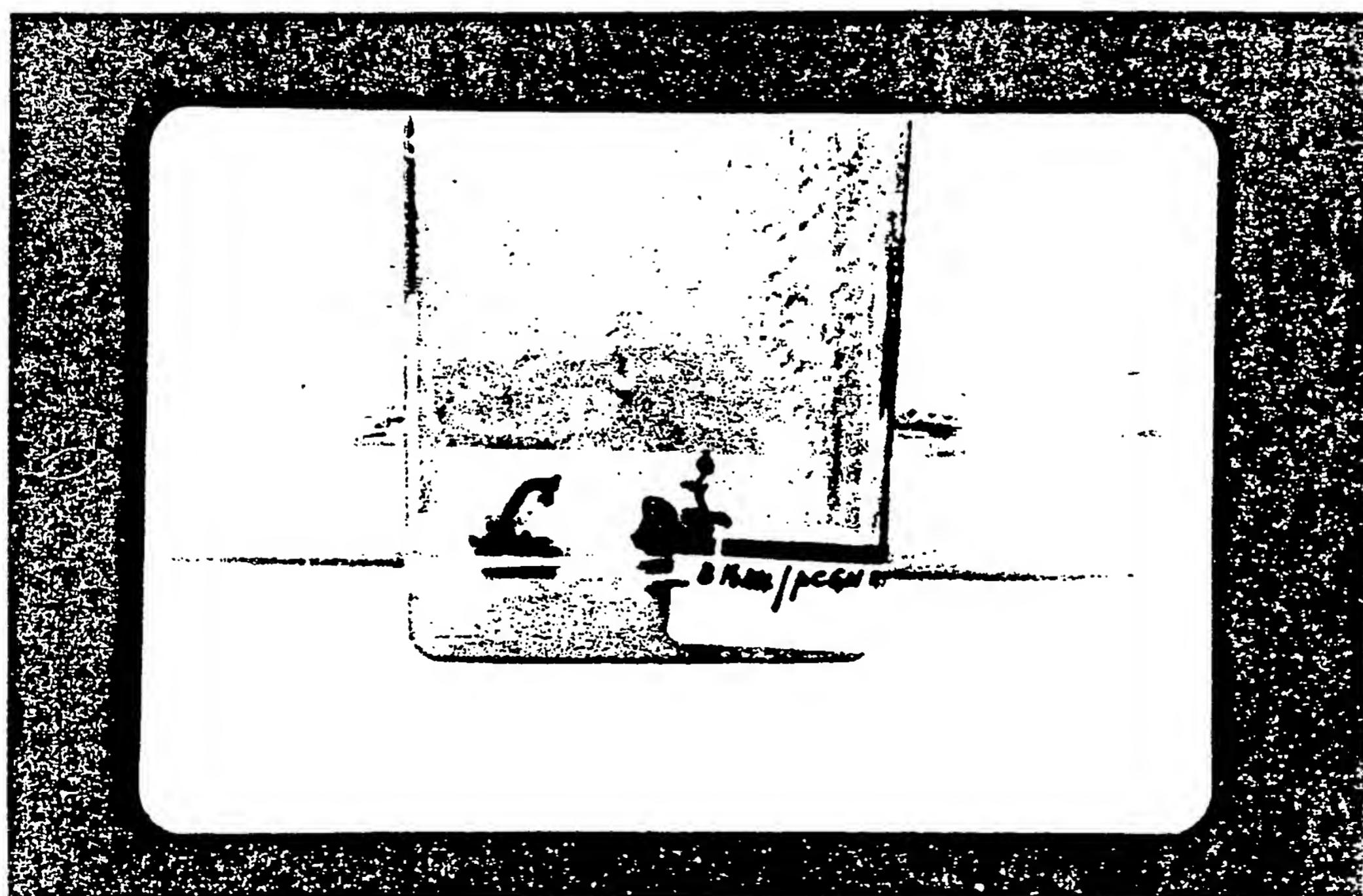


Fig. 13



Fig. 14



10 / 29

Fig. 15



Fig. 16

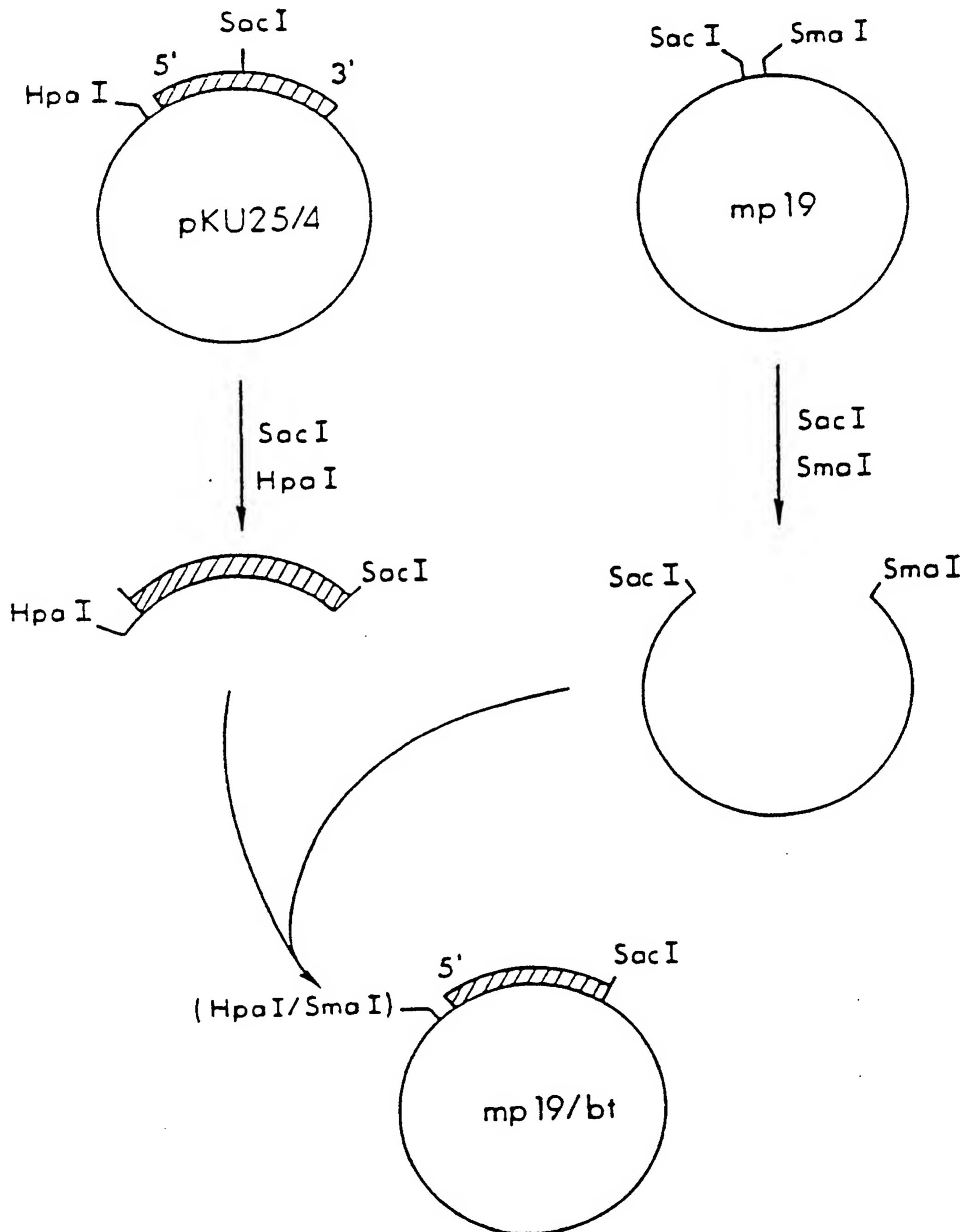


Fig. 17

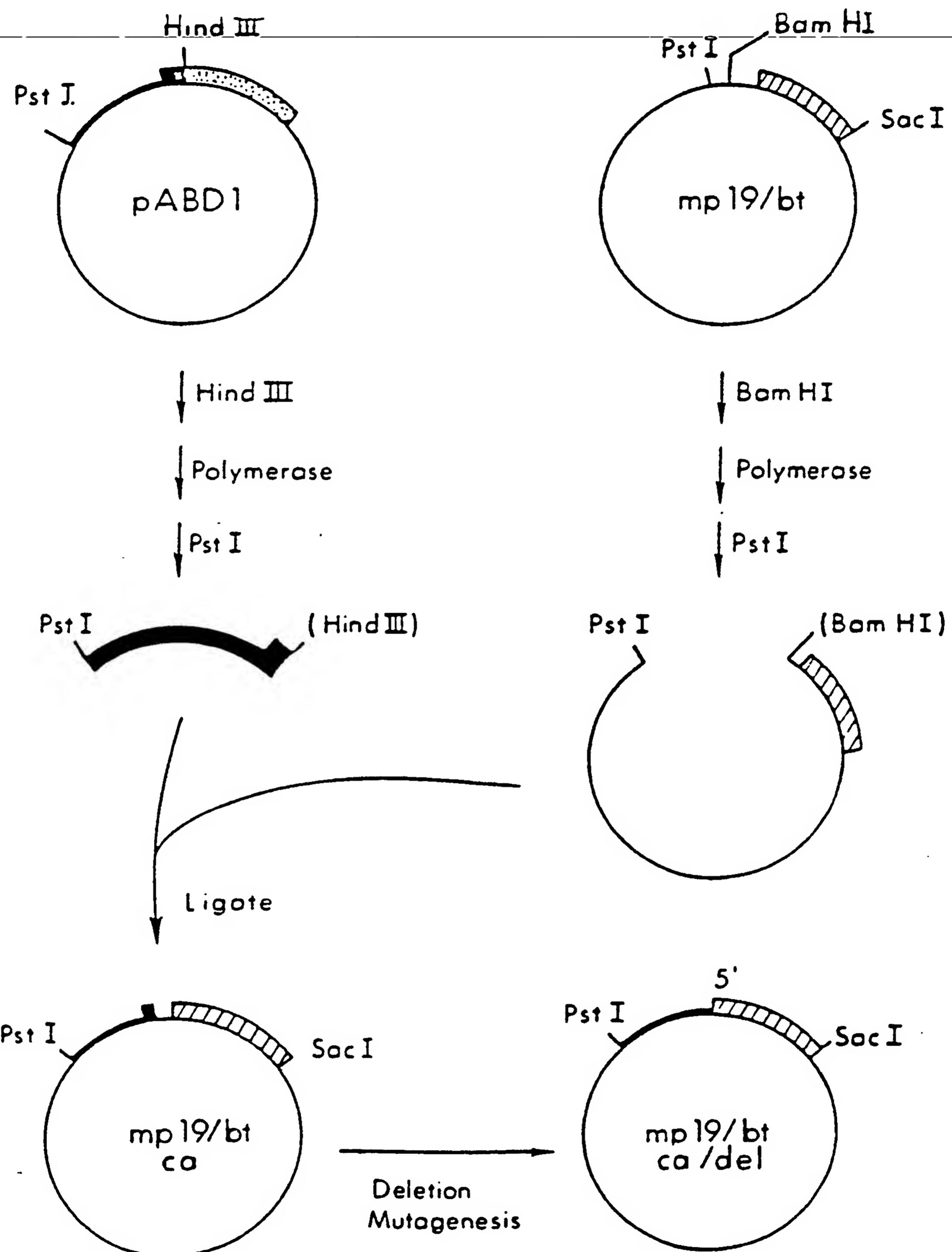


Fig. 18.

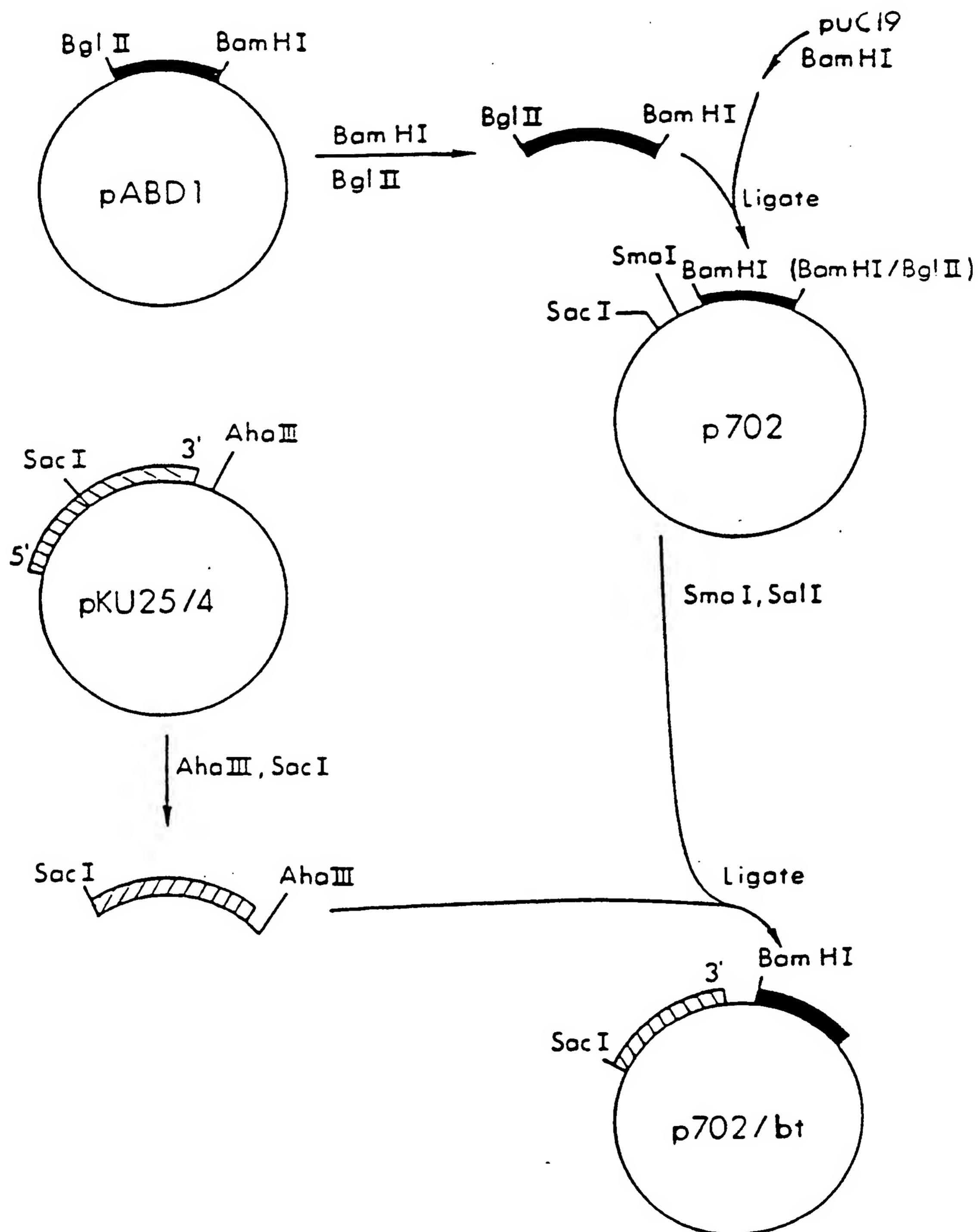


FIG. 19

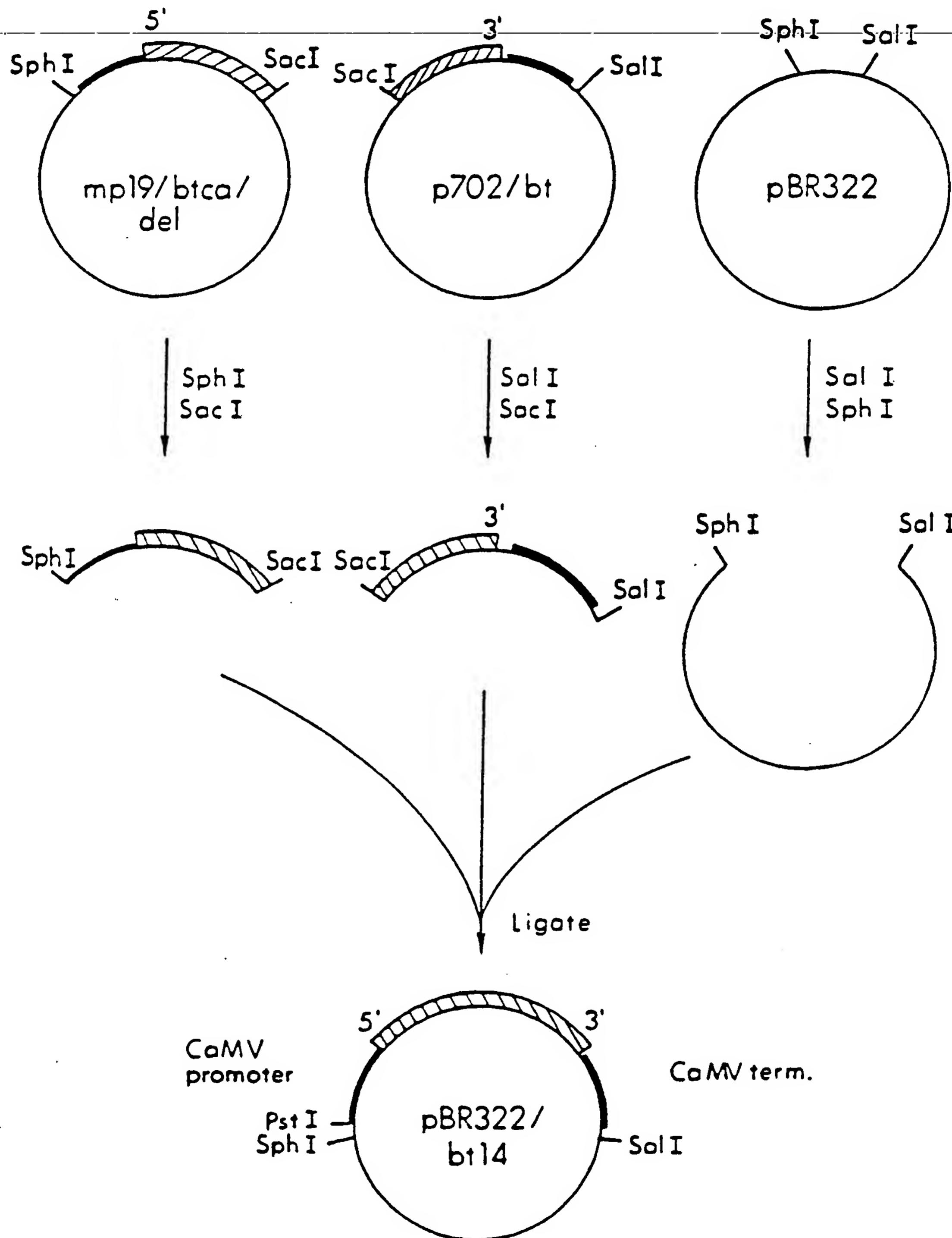


Fig. 20

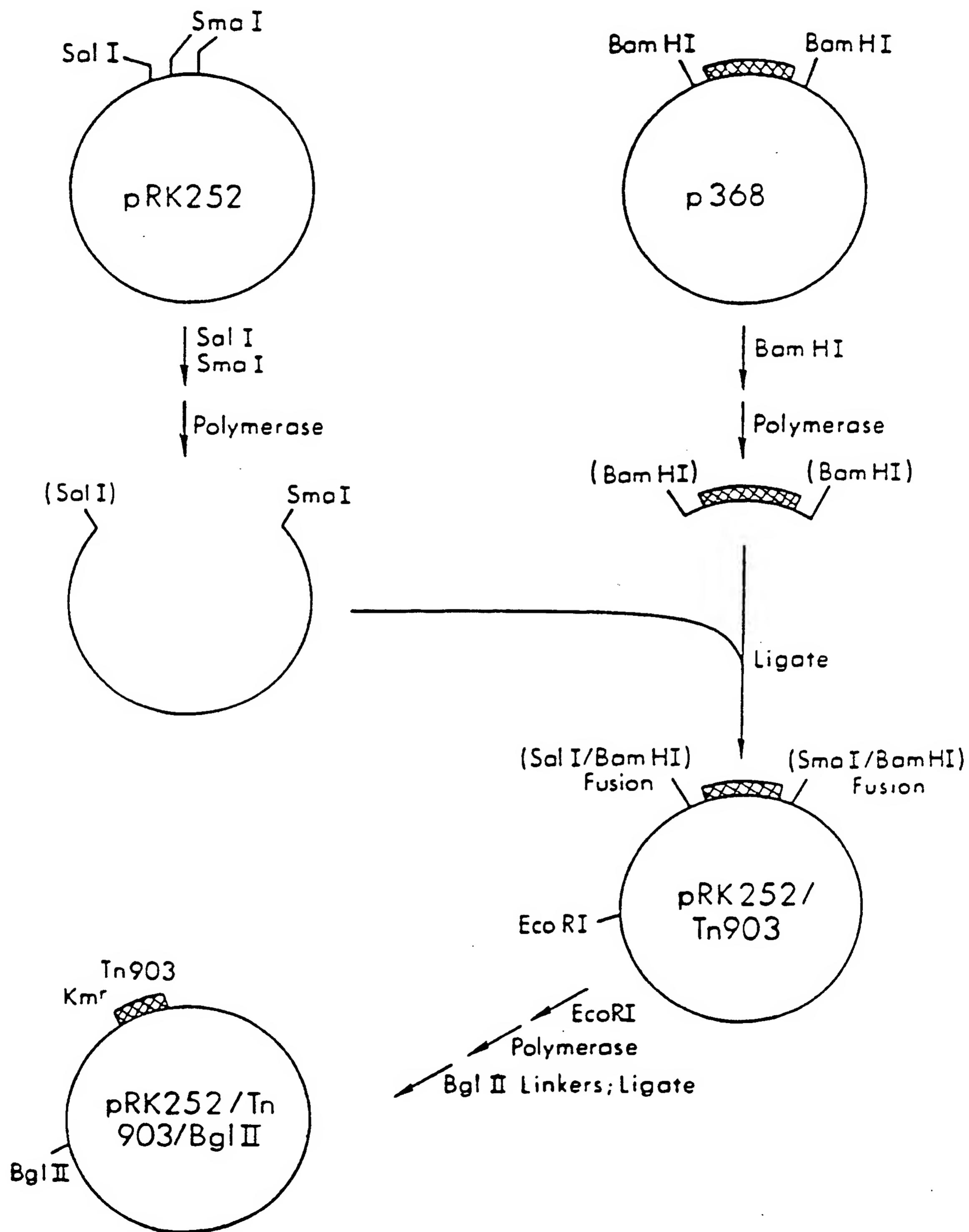


Fig. 21

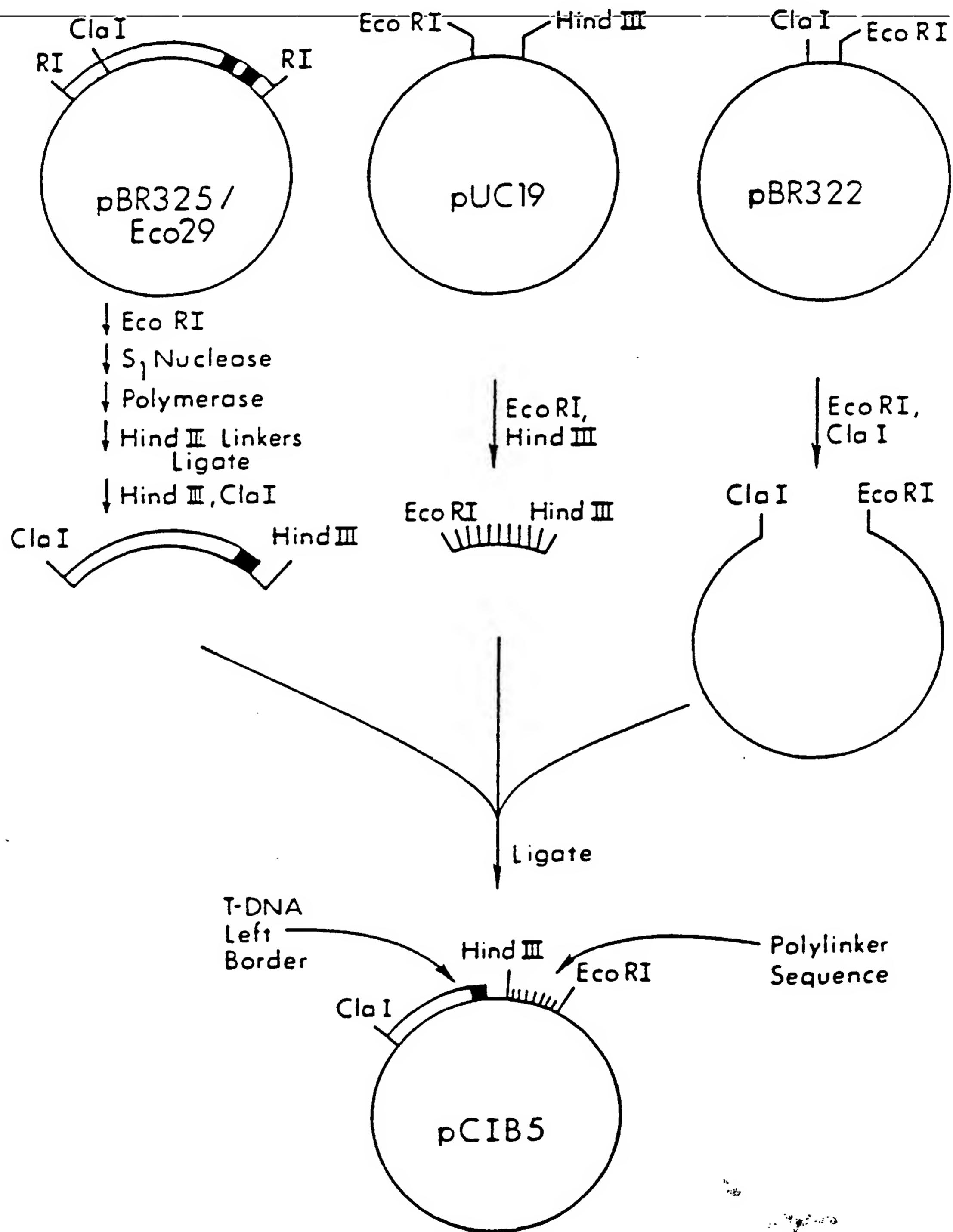


Fig. 22

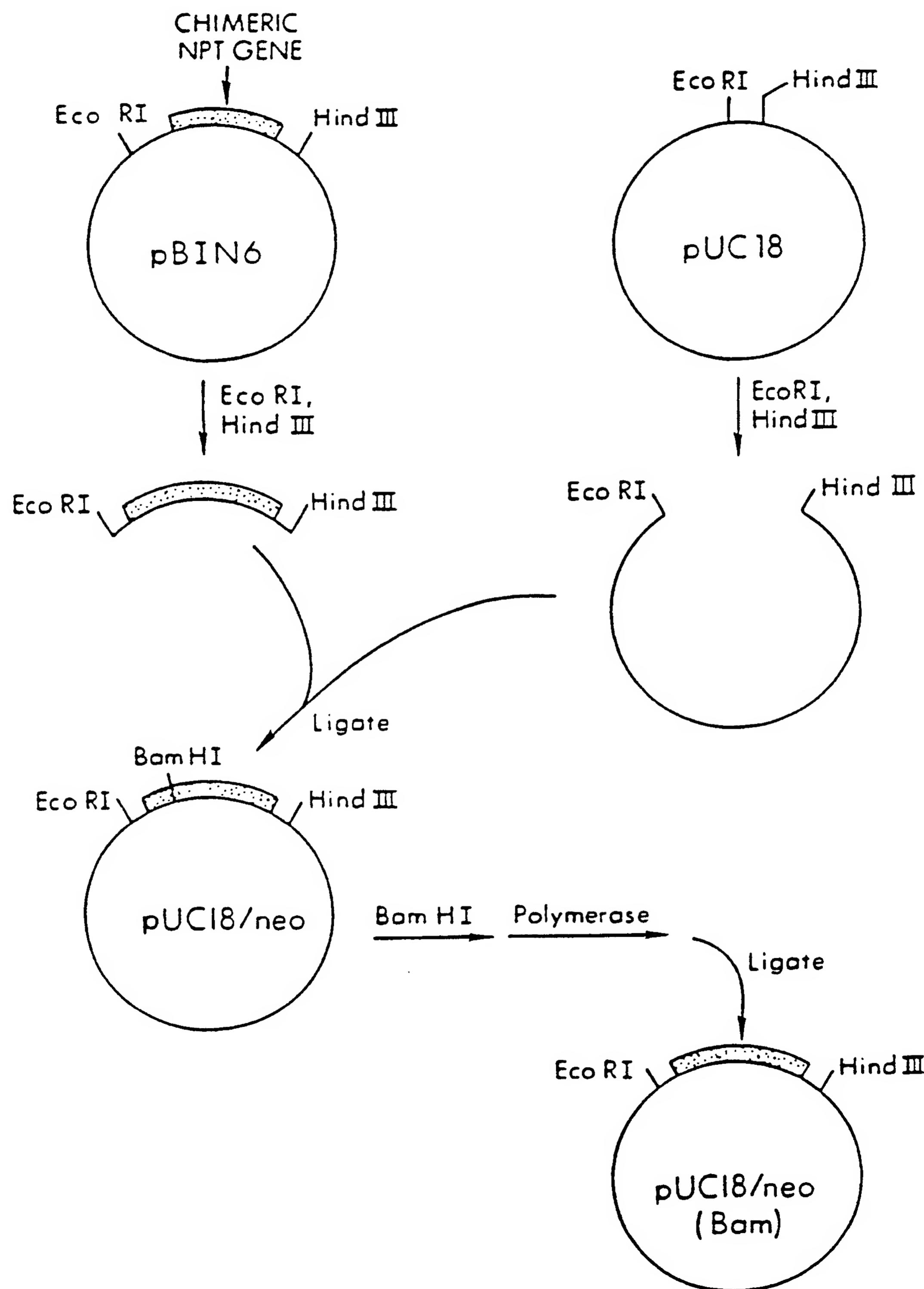


Fig. 23

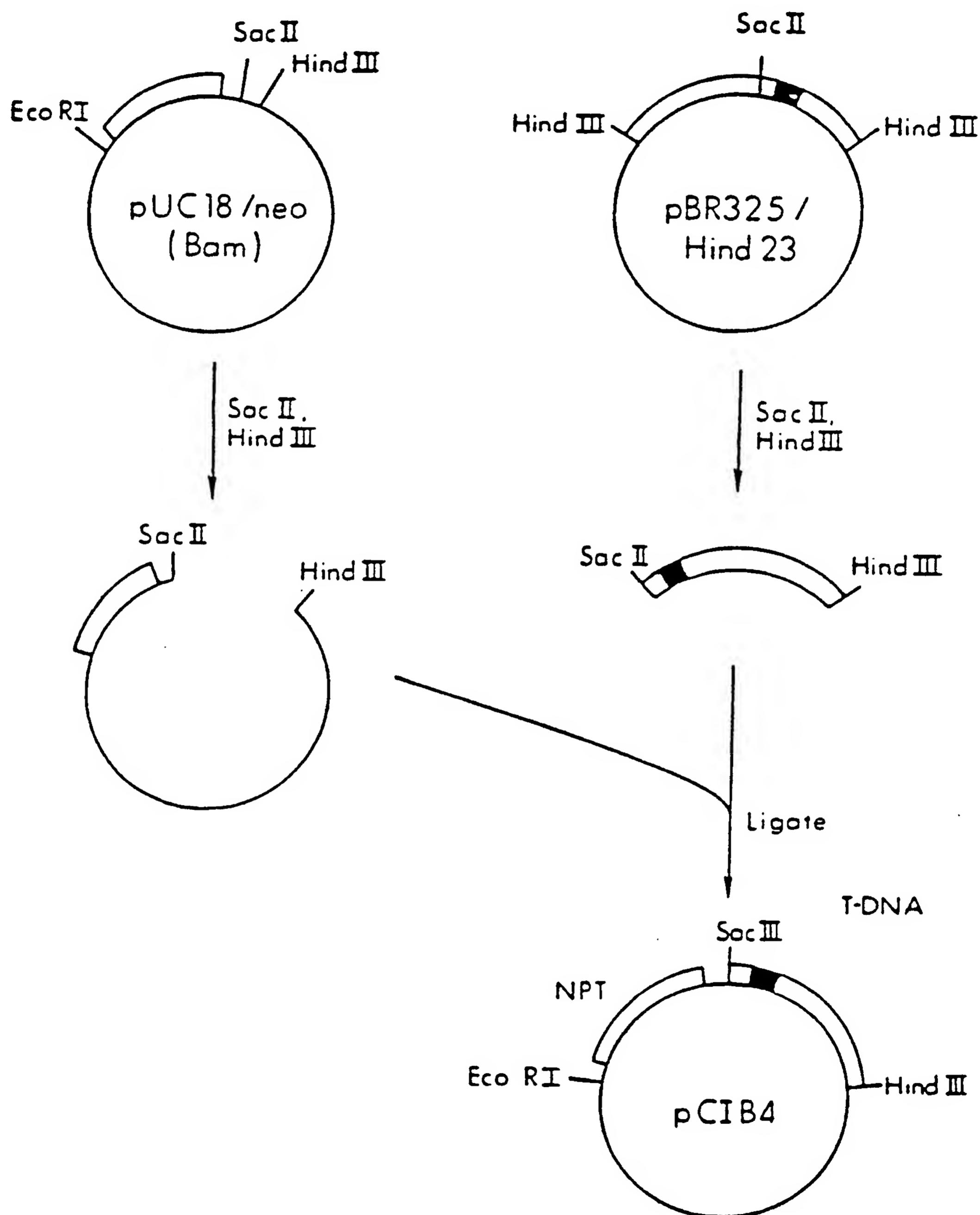


Fig. 24

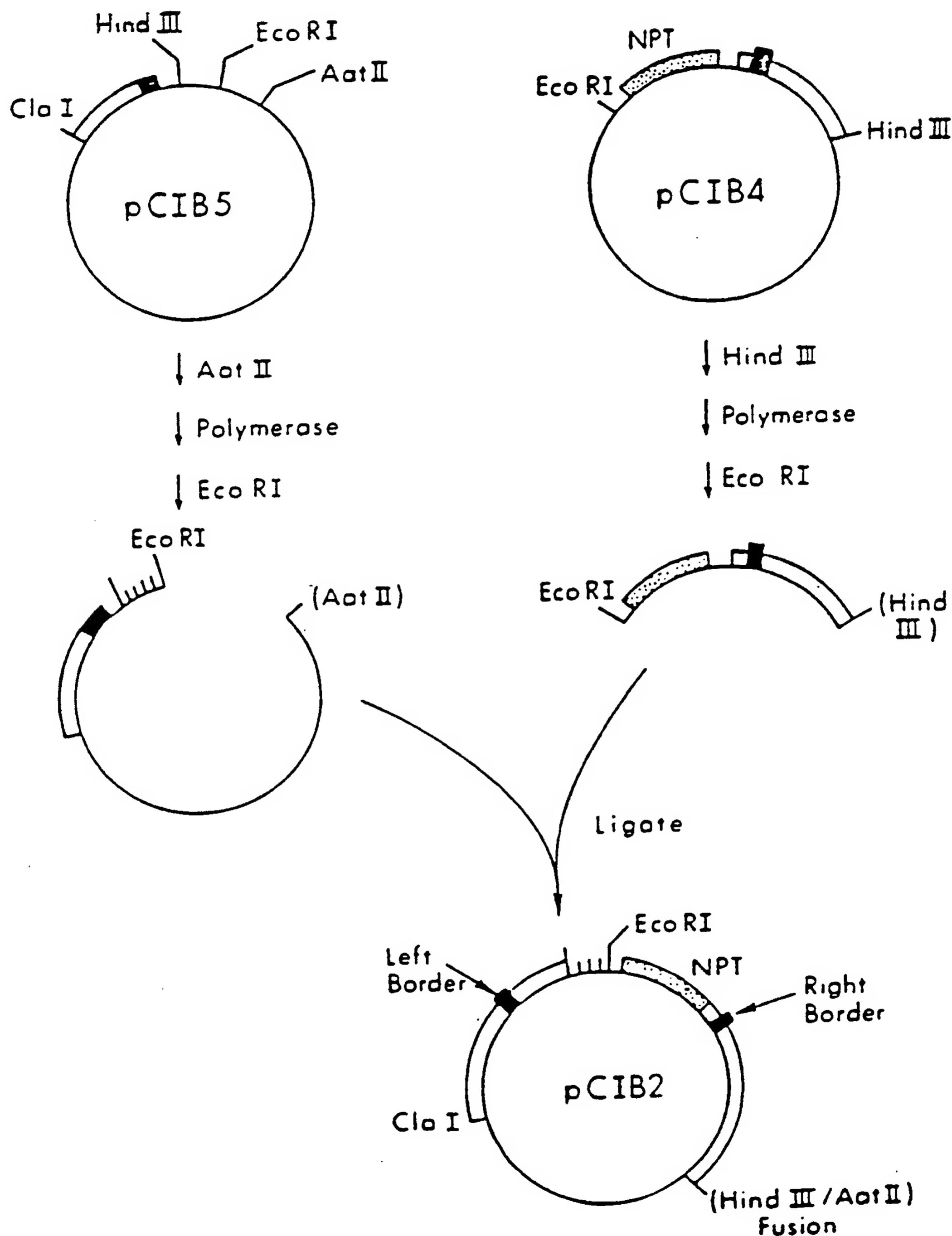


Fig. 25

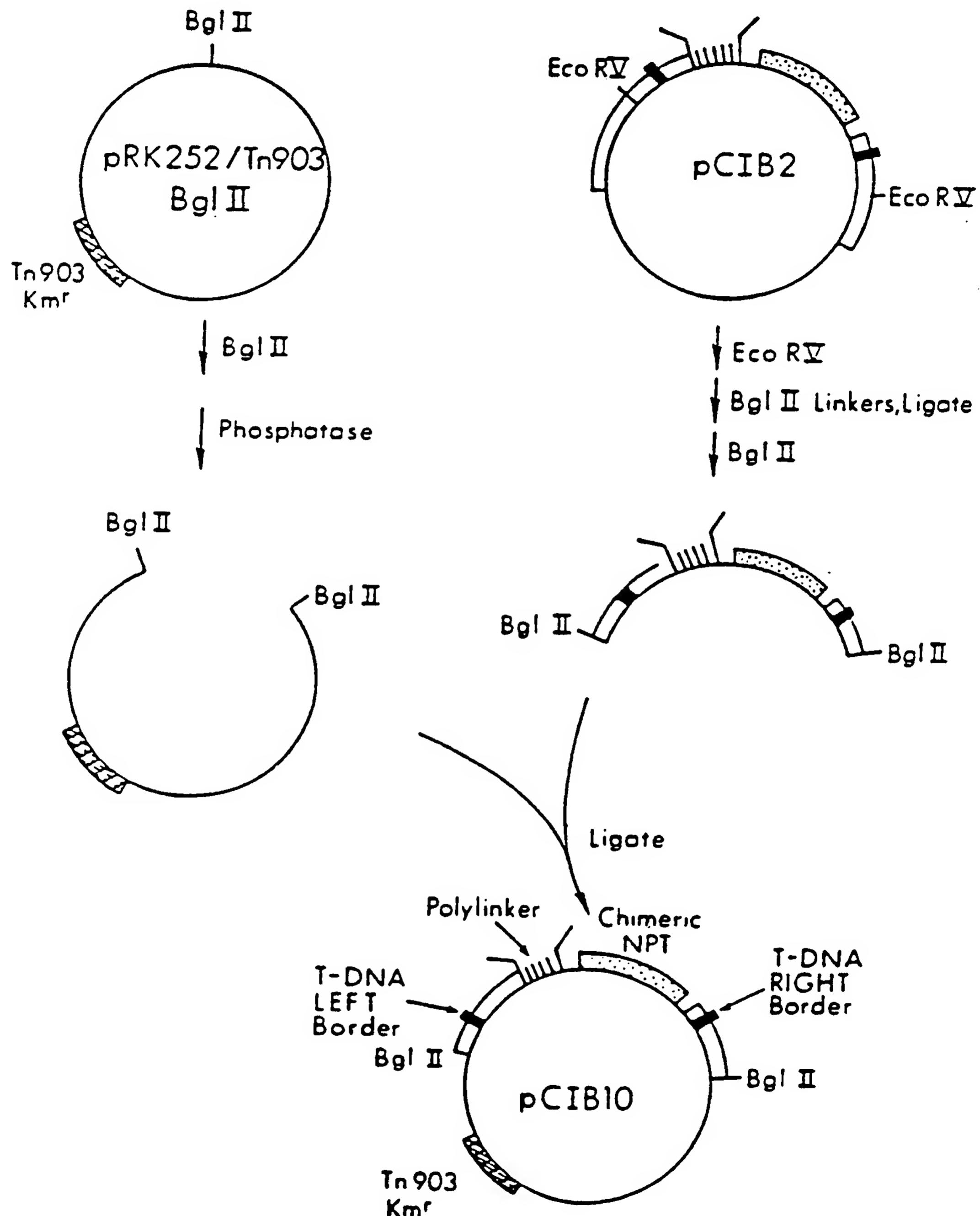


Fig. 26

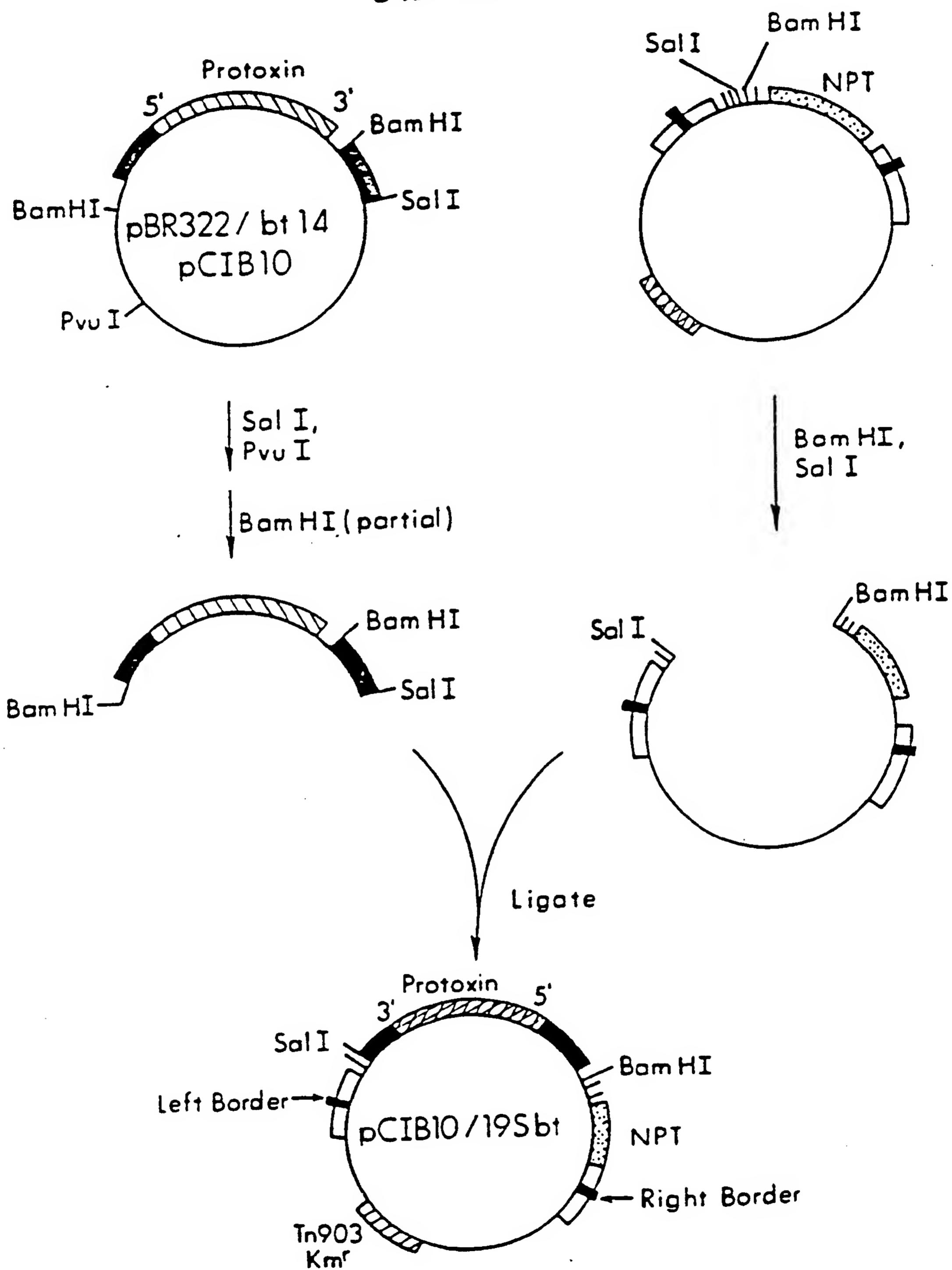


Fig. 27

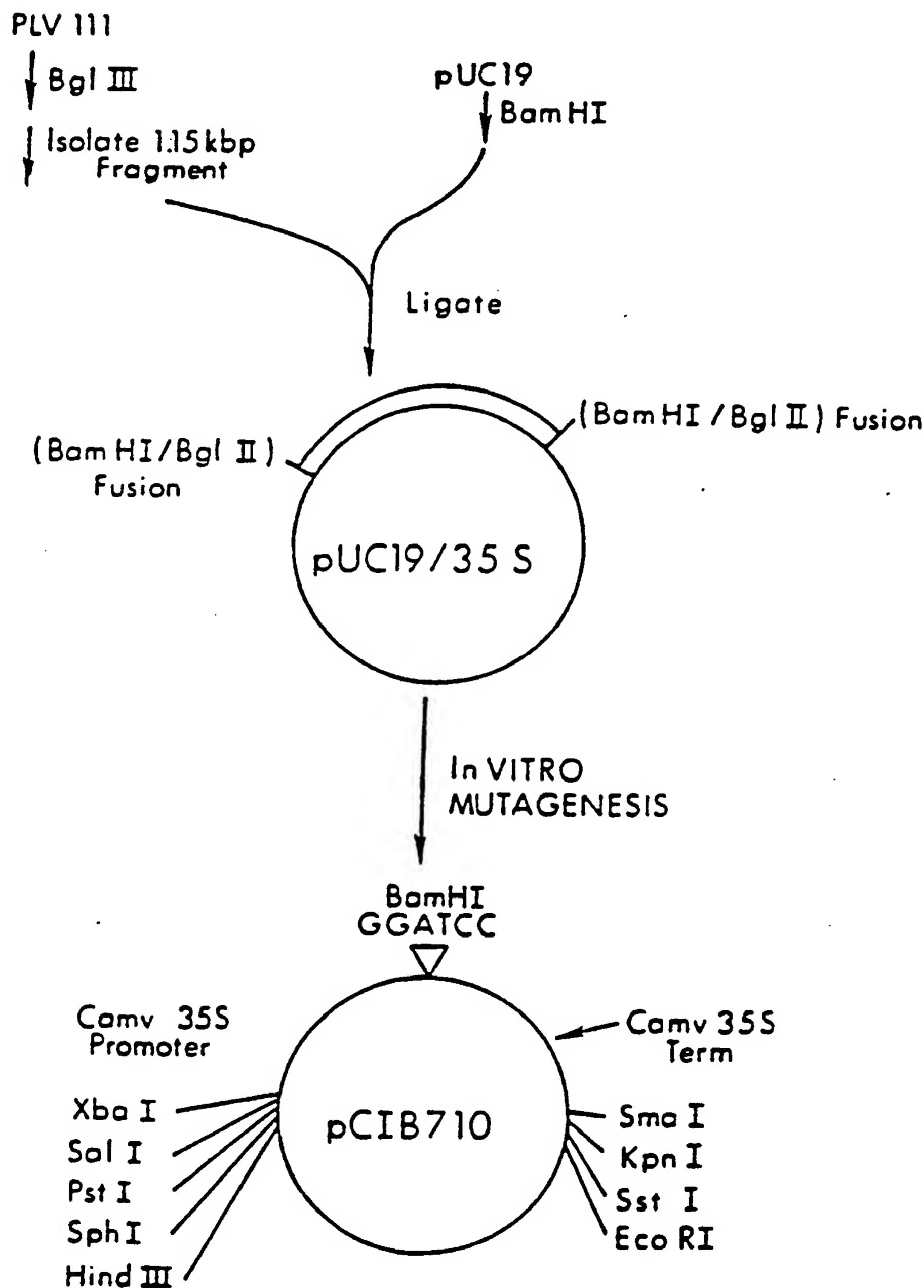


Fig. 28

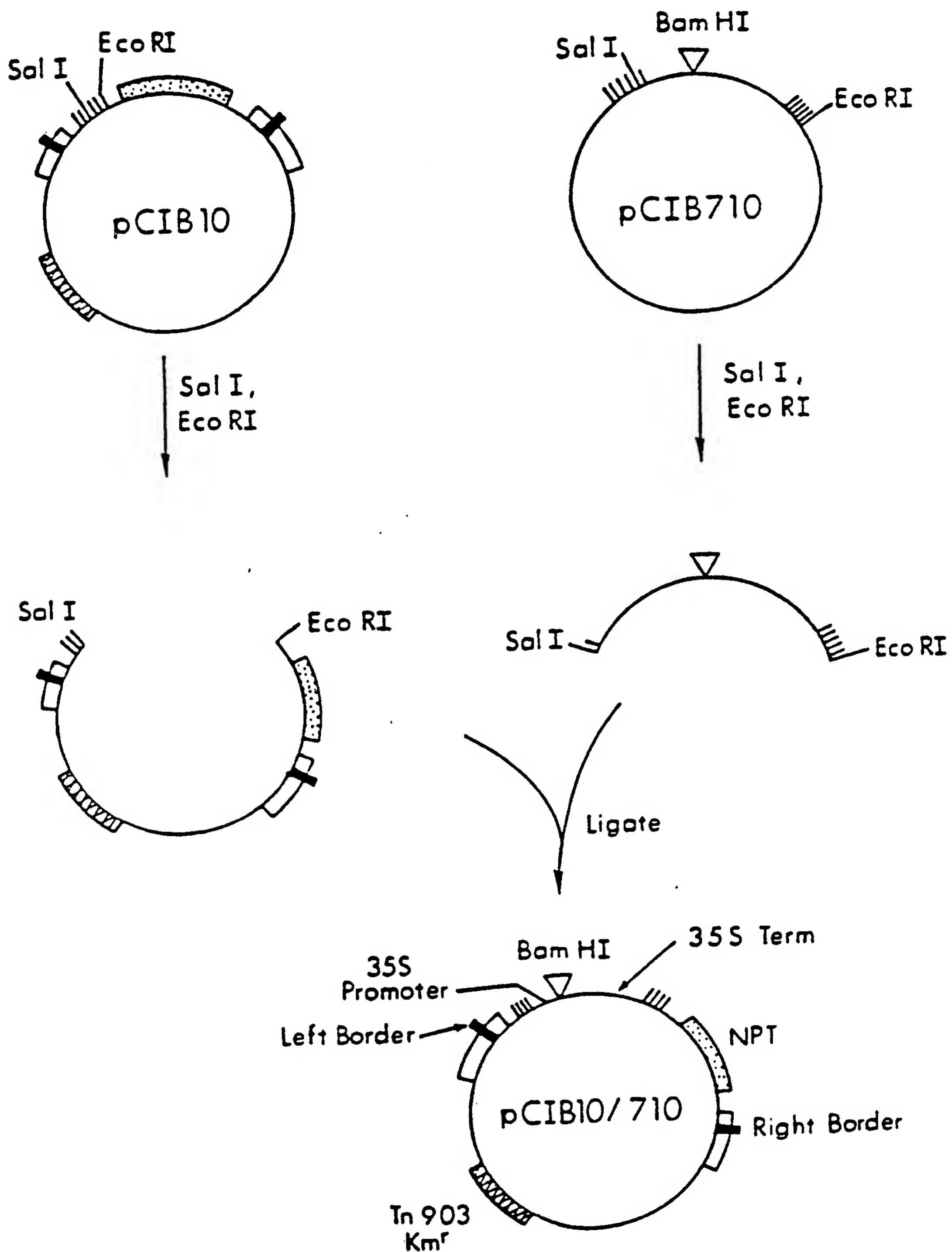
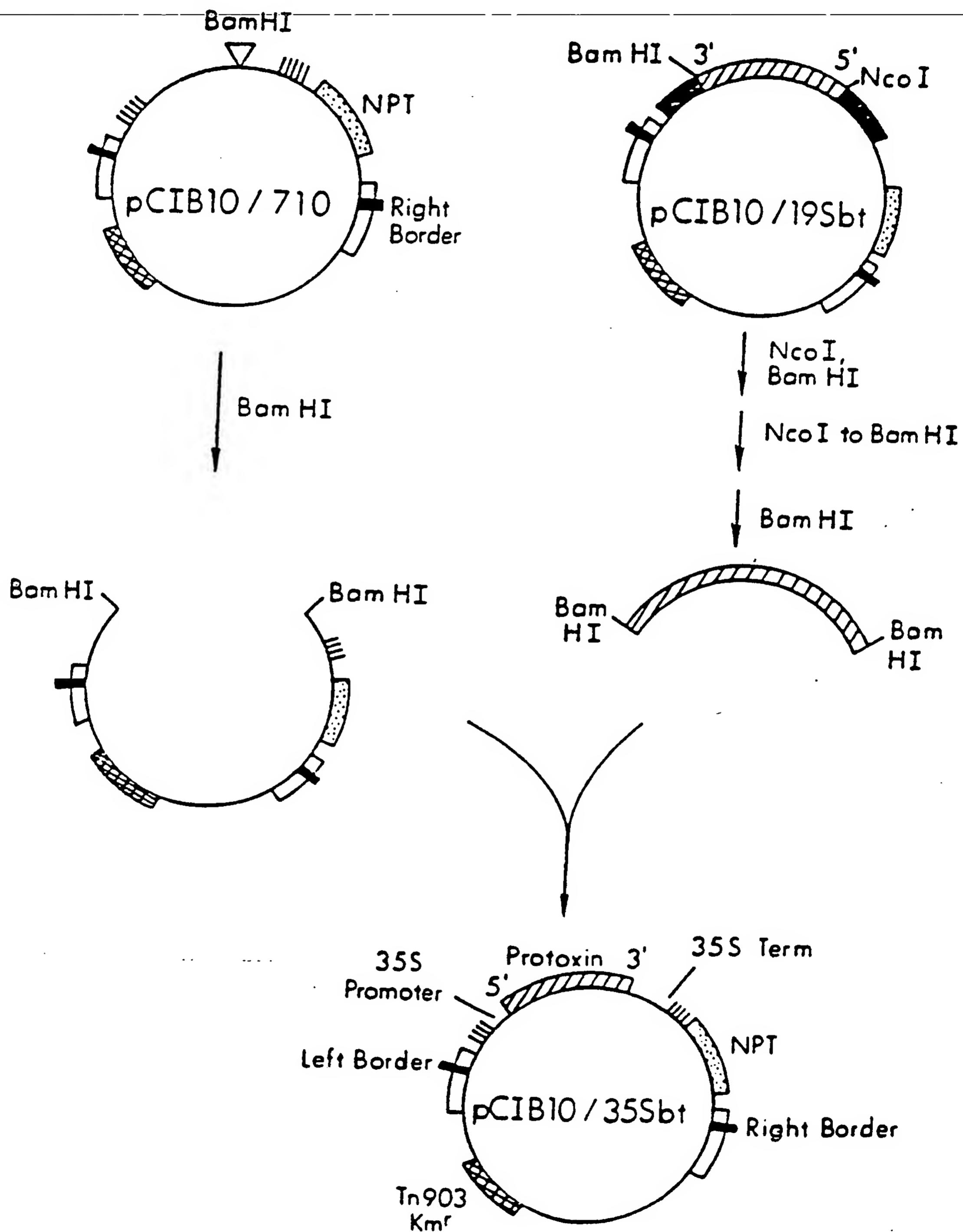


Fig. 29



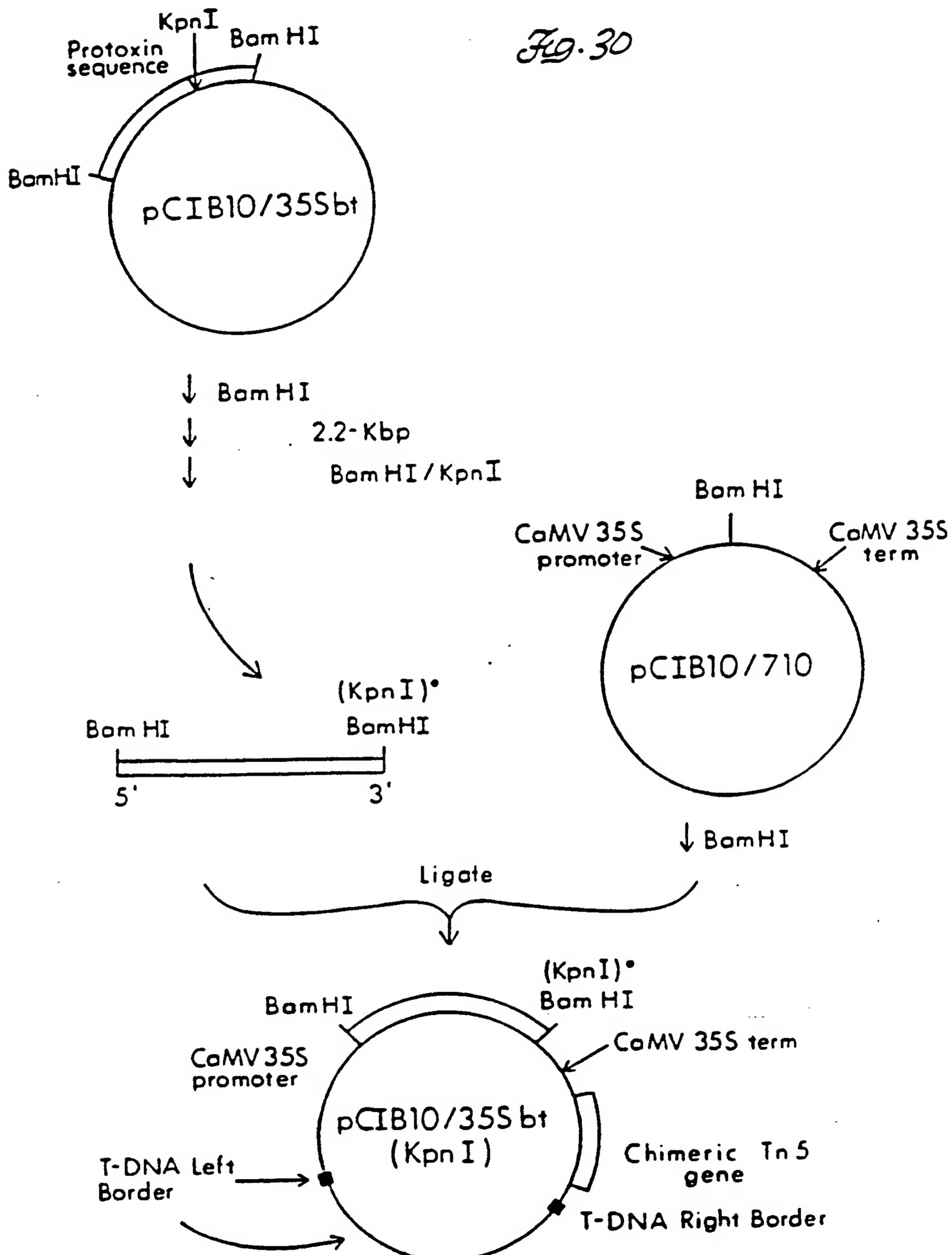


Fig. 31

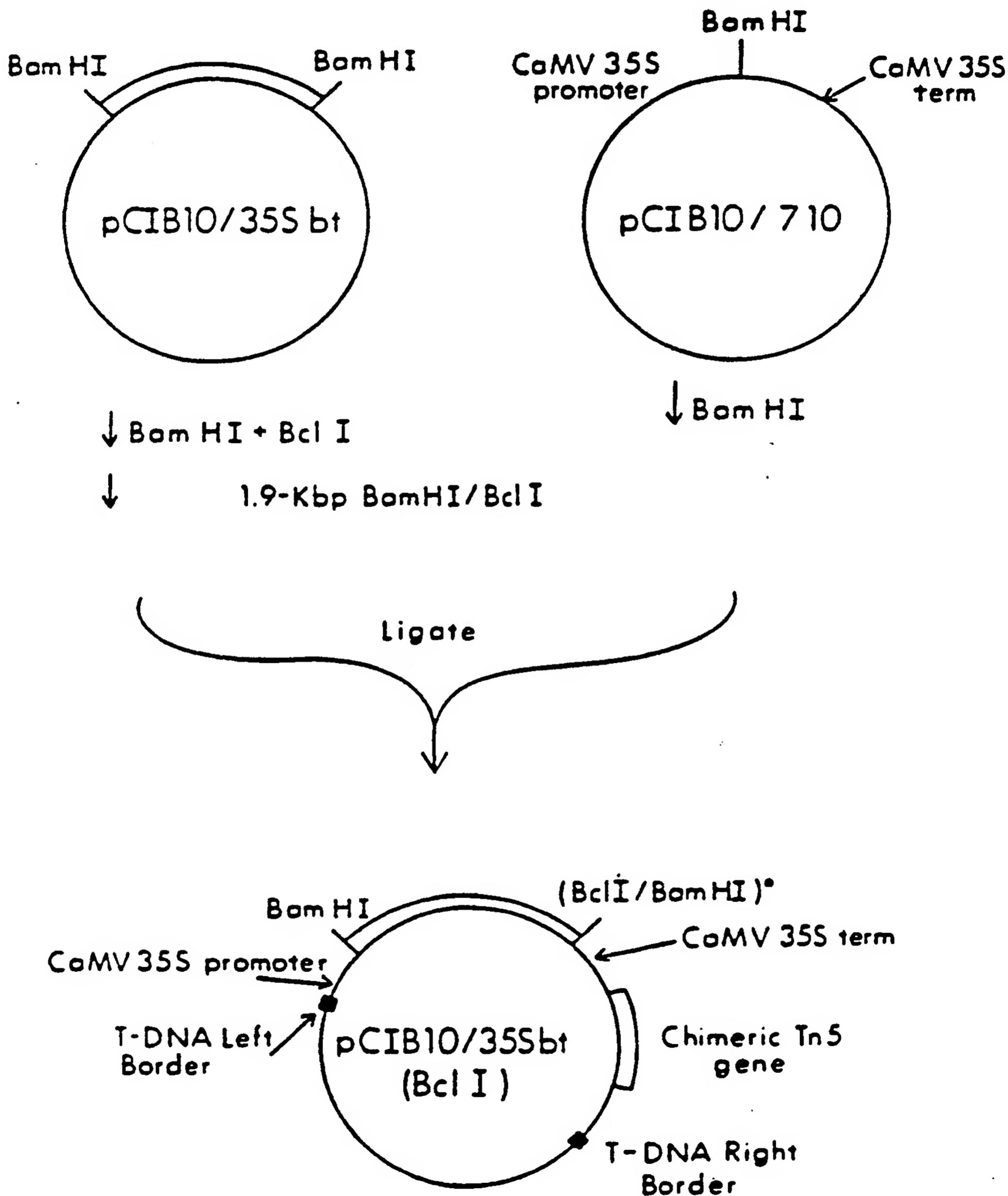
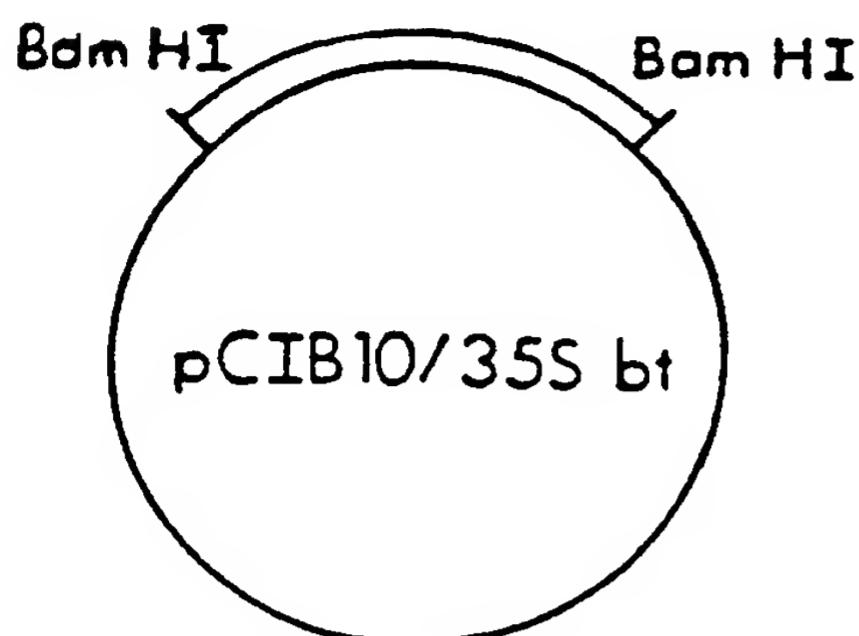
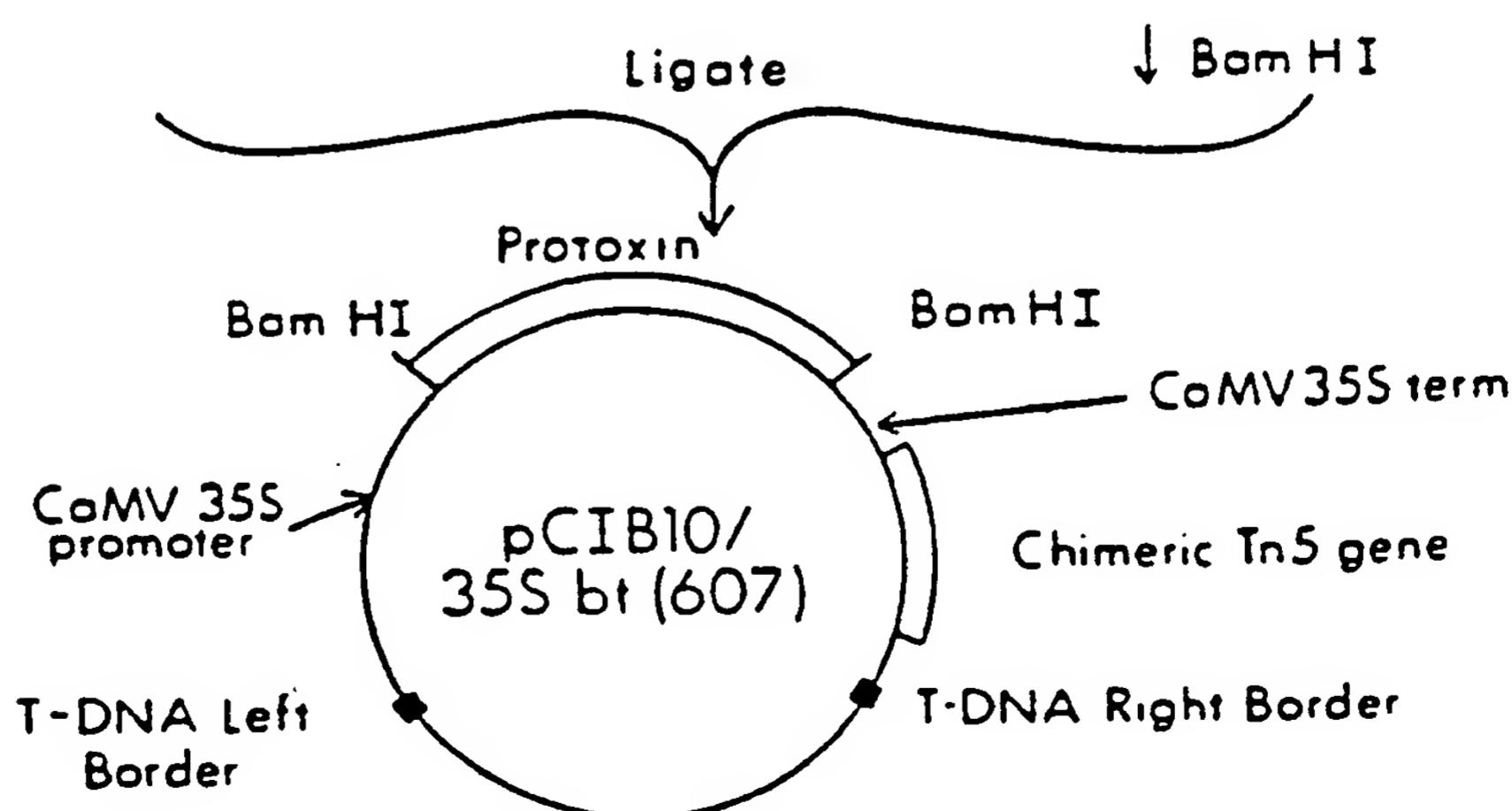
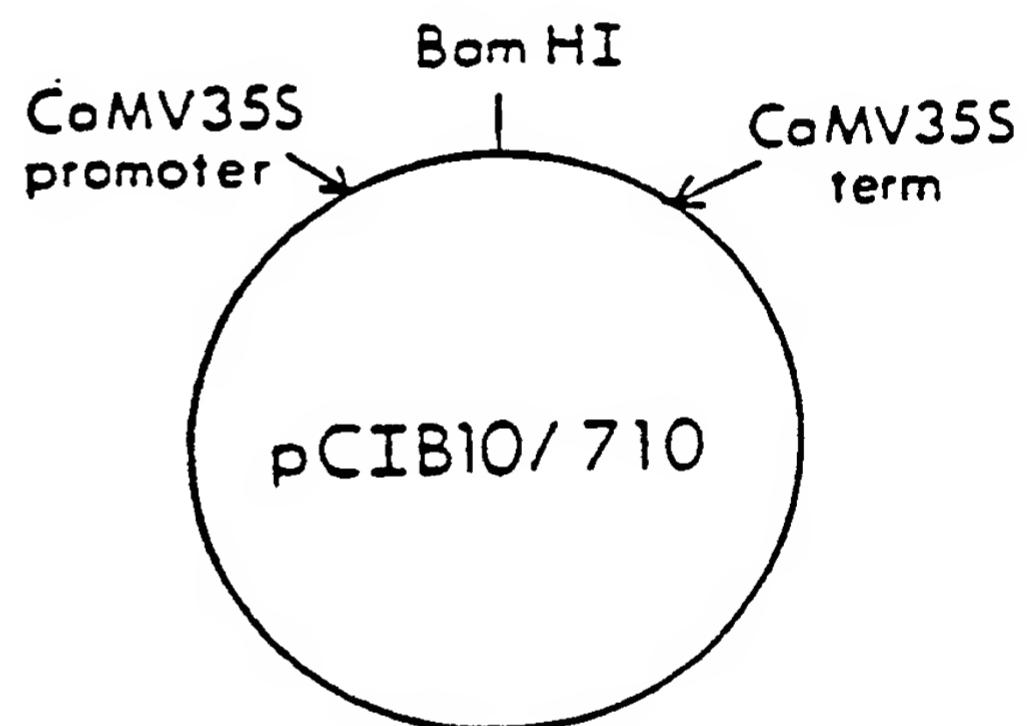
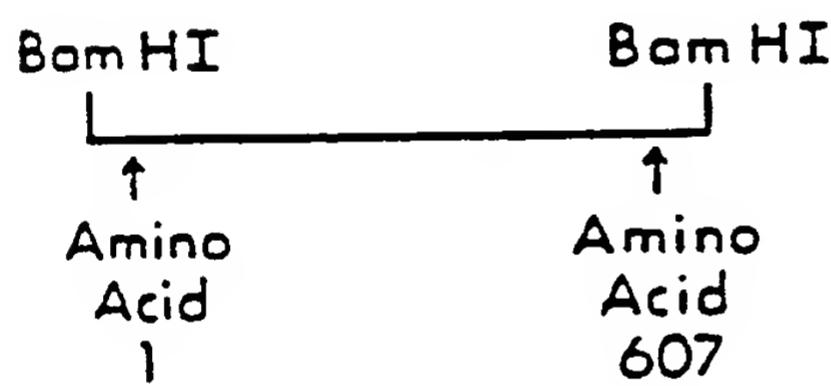


Fig. 32



↓ Bam HI
 ↓ 3.5 Kbp Bam HI
 ↓ Clone into mp18
 ↓ In vitro mutagenesis
 ↓ Cleave with Bam HI



28 / 29

Fig. 33

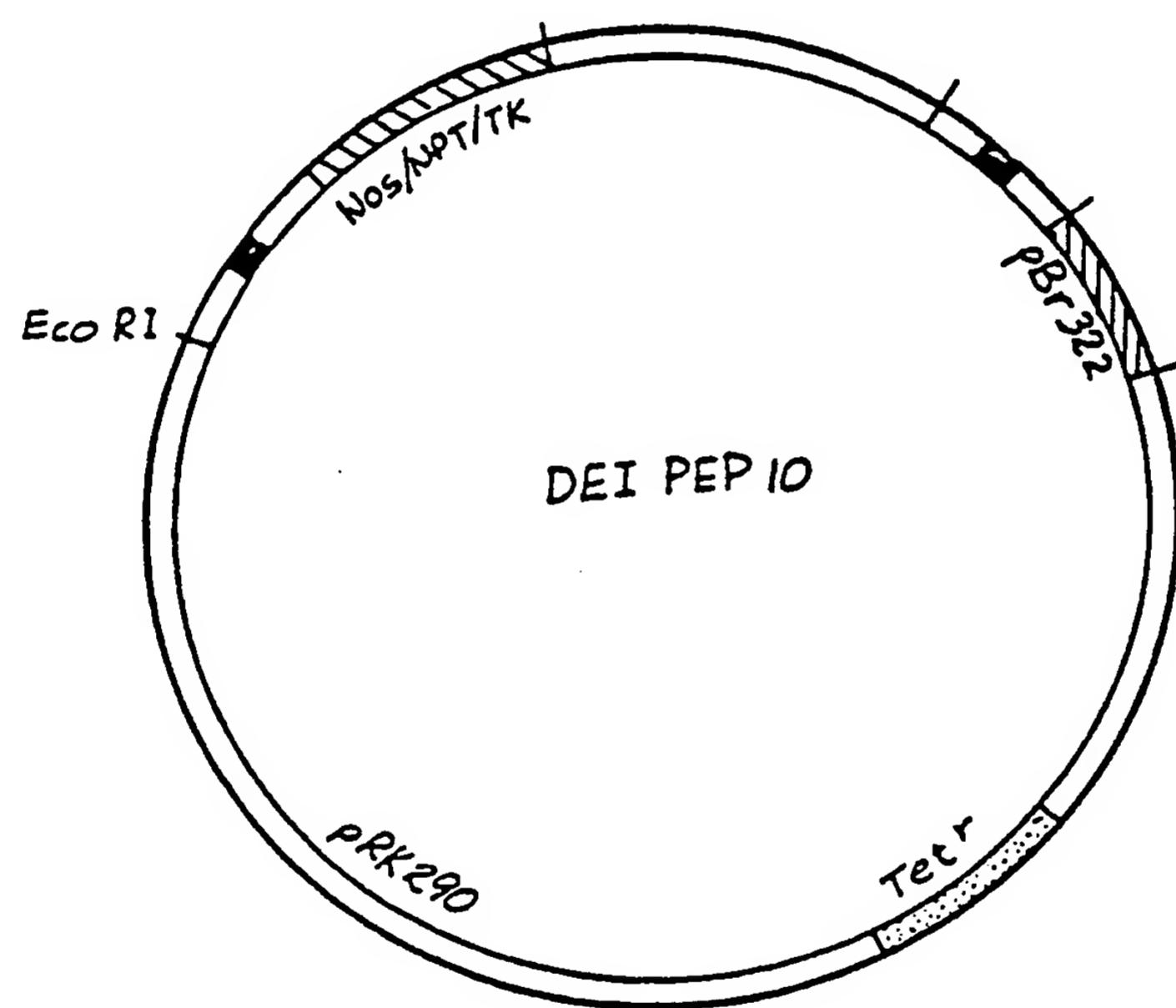


Fig. 34

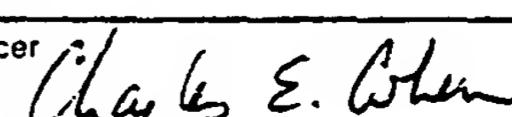


Fig. 35



INTERNATIONAL SEARCH REPORT

International Application No. PCT/U88/04116

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶					
<p>According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): C12N 5/00; C12N 15/00; A01H 17/04; U.S. Cl.: 435/240.5; 435/172.3; 800/1; 435/320; 435/240.46</p>					
II. FIELDS SEARCHED					
Minimum Documentation Searched ⁷ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 20%;">Classification System</th> <th style="width: 80%;">Classification Symbols</th> </tr> </thead> <tbody> <tr> <td style="text-align: center;">U.S.</td> <td style="text-align: center;">435/240.5; 435/172.3; 800/1; 435/240.46; 435/320</td> </tr> </tbody> </table>		Classification System	Classification Symbols	U.S.	435/240.5; 435/172.3; 800/1; 435/240.46; 435/320
Classification System	Classification Symbols				
U.S.	435/240.5; 435/172.3; 800/1; 435/240.46; 435/320				
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ <p>Databases: Chemical Abstracts Services Online (File CA, 1969-1989). File Biosis, 1969-1989). Automated Patent System (File USPAT, 1975-1989). See attachment for search terms.</p>					
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹					
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³			
X Y	<p>US, A, 4,672,035 (DAVIDONIS ET AL.) 09 June 1987. See the entire document.</p>	1,4 2,3, 5-78			
Y	<p>Chemical Abstracts, Volume 100, No. 9, issued 1984, February 27 (Columbus, Ohio, USA), Davidonis et al., "Plant regeneration from callus tissue of <i>Gossypium hirsutum L.</i>," see page 343, column 2, the abstract no. 65175p, Sci. Lett. 1983, 32(1-2): 89-93 (Eng.).</p>	1-78			
Y	<p>Biological Abstracts, Volume 78, No. 8, issued 15 October 1984 (Philadelphia, Pennsylvania, USA), Stuart et al., "Somatic embryogenesis from cell cultures of <i>Medicago sativa</i>: 2: The interaction of amino acids with ammonium," see page 6339, column 1, the abstract no. 56366, Plant Sci. Lett. 1984, 34(1/2): 175-182 (Eng.).</p>	1-78			
<p>[*] Special categories of cited documents: ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed</p> <p>¹¹ "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family</p>					
IV. CERTIFICATION					
Date of the Actual Completion of the International Search 24 March 1989	Date of Mailing of this International Search Report 04 MAY 1989				
International Searching Authority ISA/US	Signature of Authorized Officer  Charles E. Cohen				

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____, because they relate to subject matter¹² not required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹³, specifically:

3. Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Trolinder et al., <u>Proceedings of the Beltwide Cotton Production Research Conferences</u> , January 6-11, 1985, New Orleans, LA., page 46.	1-78
Y	Biological Abstracts, Volume 82, No. 9, issued 01 November 1986, (Philadelphia, Pennsylvania, USA), Shoemaker et al., "Characterization of somatic embryogenesis and plant regeneration in cotton (<i>Gossypium hirsutum</i> L.)," see page AB-907, Column 2, the abstract no. 86768, <i>Plant Cell Rep.</i> 1986, 5(3): 178-181 (Eng.).	1-78
Y	Chemical Abstracts, Volume 106, No. 9, issued 1987, March 02 (Columbus, Ohio, USA), N.L.G. Trolinder, "Somatic embryogenesis and plant regeneration in <i>Gossypium hirsutum</i> L.," see page 225, column 1, the abstract no. 62935z, <i>Diss. Abstr. Int. B</i> 1986, 47(6): 2250-1 (Eng.).	1-78
Y	Chemical Abstracts, Volume 107, No. 5, issued 1987, August 03 (Columbus, Ohio, USA), Trolinder et al., "Somatic embryogenesis and plant regeneration in cotton (<i>Gossypium hirsutum</i> L.)," see page 369, column 1, the abstract no. 36159a, <i>Plant Cell Rep.</i> 1987, 6(3): 231-4 (Eng.).	1-78
Y	Chemical Abstracts, Volume 106, No. 21, issued 1987, May 25 (Columbus, Ohio, USA), Umbeck et al., "Genetically transformed cotton (<i>Gossypium hirsutum</i> L.) plants," see page 179, column 1, the abstract no. 170182k, <i>Bio/Technology</i> 1987, 5(3): 263-6 (Eng.).	58-79, 85-86
Y	Chemical Abstracts, Volume 108, No. 11, issued 1988, March 14 (Columbus, Ohio, USA), Firoozabady et al., "Transformation of cotton (<i>Gossypium hirsutum</i> L.) by <i>Agrobacterium tumefaciens</i> and regeneration of transgenic plants," see page 153, column 1, the abstract no. 88857s, <i>Plant Mol. Biol.</i> 1987, 10(2): 105-16 (Eng.).	58-79, 85-86

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The EMBO Journal, Volume 3, No. 8, issued August 1984, (Oxford, England), De Block et al., "Expression of foreign genes in regenerated plants and in their progeny" pages 1681-1689. See entire document	58-79, 85-86
Y	Chemical Abstracts, Volume 98, No. 23, issued 1983, June 6 (Columbus, Ohio, USA), Becker et al., "Herpes simplex virus type 1 thymidine kinase gene expression in Escherichia coli, "see page 168, column 2, the abstract no. 192666q, Gene 1983, 21(1-2): 51-8 (Eng.).	86
Y	Chemical Abstracts, Volume 100, No. 9, issued 1984, February 27 (Columbus, Ohio, USA), Kit et al., "Nucleotide sequence of the herpes simplex virus type 2 (HSV-2) thymidine kinase gene and predicted amino acid sequence of thymidine kinase polypeptide and its comparison with the HSV-1 thymidine kinase gene," see page 131, column 1, the abstract no. 62615w, Biochim. Biophys. Acta 1983, 741(2): 158-70 (Eng.).	86
X	Chemical Abstracts, Volume 100, No. 15, issued 1984, April 9 (Columbus, Ohio, USA), N. Sh. Alieva, "Physiological and biochemical characteristics of cotton varieties differing in their resistance to wilt," see page 327, column 1, the abstract no. 117970r, K. Izuch. Rezist. Rast. Ekstremal'nykh Vozdeistv. Sredy 1982, 44-51 (Russ).	80,84
X:	Chemical Abstracts, Volume 103, No. 11, issued 1985, September 16 (Columbus, Ohio, USA), Mace et al., "Toxicity and role of terpenoid phytoalexins in verticillium wilt resistance in cotton," see page 379, column 1, the abstract no. 85240w, Physiol. Plant Pathol. 1985, 26(2): 209-218 (Eng.).	80,84

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Chemical Abstracts, Volume 100, No. 23, issued 1984, June 04 (Columbus, Ohio, USA), Khamraev et al., "Potassium fertilization: yield and quality," see page 499, column 2, the abstract no. 190836f, Khlopkovodstvo 1984, 3: 24-5 (Russ.).	81
X	Chemical Abstracts, Volume 105, No. 8, issued 1986, August 25 (Columbus, Ohio USA), Sarmina et al., "Effect of exposure to energy on the physicomechanical properties of cotton fibers treated with trace elements," see page 76, column 1, the abstract no. 62035k, Izv. Akad. Nauk Tadzh. SSR, Otd. Fiz.-Mat., Khim. Geol. Nauk 1985, 4: 20-5 (Russ.).	81
X	Chemical Abstracts, Volume 96, No. 15, issued 1982, April 12 (Columbus, Ohio, USA), Ryan et al., "The metabolism of chlortoluron, diuron, and CGA 43 057 in tolerant and susceptible plants," see page 236, column 1, the abstract no. 117559n, Pestic. Biochem. Physiol. 1981, 16(3): 213-21 (Eng.).	82
X	Chemical Abstracts, Volume 100, No. 11, issued 1984, March 12 (Columbus, Ohio, USA), Khuzhanazarov et al., "Effect of copper and zinc in basic fertilizer on the productivity of thin-fibered cotton," see page 468, column 1, the abstract no. 84740k, Khim. Sel'sk Khoz. 1983, 12: 21-2 (Russ.).	83

Attachment to Form PCT/ISA/210
Part II. FIELDS SEARCHED TERMS

Cotton
Gossypium
regenerat?
somatic
transform?
Agrobacterium
resist or toleran?
verticillium, improve?
or superior
fiber#
or fibre#
plant
herbicide#
increas?
improv?
yield#
thymidine
kinase
gene#
terminat?
inventors names

Attachment to Form PCT/ISA/210Part VI. OBSERVATIONS WHERE UNITY OF INVENTION IS
LACKING

The claims of these eight groups have the characteristics of eight distinct inventive concepts. The methods of groups I and II are distinct in that they recite different process steps and operating parameters, and produce different end products. The products of groups IV-VII are distinct from the method of group I in that plant products with the recited properties can be produced by other processes, such as by chemical or physical mutagenesis, followed by selection. The method of group I can also produce plants with a wide variety of traits other than those of the plants of these groups. The product of group III is distinct from the method of group II in that antibiotic resistant plants can be produced by methods other than transformation, such as by chemical or physical mutagenesis, and selection. The products of groups III-VII are distinct in that each possesses a unique phenotype. Finally, the product of group VIII is distinct from the method of group II in that the vectors recited can be used to transform a wide variety of plants other than cotton, and in that a wide variety of other vector constructs can be employed in the method of group II to produce transformed cotton plants.

Continuation to Attachment Form PCT/ISA/210,
Part VI. OBSERVATION WHERE UNITY OF INVENTION IS
LACKING:

Group I: Claims 1-57, drawn to a method for regenerating cotton from explant material via callus; Class 435/240.5.

Group II: Claims 58-78, drawn to a method for transforming cotton; Class 435/172.3.

Group III: Claim 79, drawn to antibiotic resistant cotton plants; Class 800/1.

Group IV: Claims 80 and 84, drawn to fungal resistant cotton plants; Class 800/1.

Group V: Claim 81, drawn to cotton plants with improved fiber; Class 800/1.

Group VI: Claim 82, drawn to cotton plants exhibiting herbicide tolerance; Class 800/1.

Group VII: Claim 83, drawn to cotton plants exhibiting increased yield; Class 800/1.

Group VIII: Claim 85-86, drawn to a transformation vector; Class 435/320.